

FINAL REPORT

FOR

A STUDY TOWARD DEVELOPMENT OF AN
AUTOMATED MICROBIAL METABOLISM LABORATORY

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ABSTRACT

Metabolic parameters **for** life detection have been successfully demonstrated by phosphate uptake, sulfate uptake, and ATP production of Earth-born microorganisms using RM9- $^{35}\text{SO}_4$ -S medium. Phosphate uptake was determined by ~~measuring~~ decrease of phosphate concentration in supernatant of the test medium. Sulfate uptake ~~was~~ shown by accumulation of $^{35}\text{SO}_4$ -S in cells. ATP production **was** demonstrated by ATP assay of the cells extracted by DMSO through subsequent injection of the extracted ATP into firefly luciferase, luciferin, and other reagents **for** light response.

Samples for detection were introduced into an experimental medium and a poisoned control. Positive response of the former in contrast to little or no response of the latter in manifesting the activities presented the evidence of microbial metabolism and life.

Analytical methods for phosphate determination included the conventional ammonium molybdate-stannous chloride method, the ^{14}C -triethylamine-phosphomolybdate method, the calcium electrode method, and enzymatic orthophosphate analysis. The last two methods were not successful. The most promising one was ^{14}C -triethylamine-phosphomolybdate method. Most of the phosphate uptake laboratory studies, however, were done by ammonium molybdate-stannous chloride method since ^{14}C -triethylamine is too expensive to be used for routine analysis. For sulfate uptake, cells after being exposed to RM9- $^{35}\text{SO}_4$ -S medium were filtered and counted by a radioactive counter. These cells were then extracted **for** ATP determination using the firefly enzymatic method. Initially, phosphate and sulfate uptake studies were done individually while the ATP study was tested along with phosphate uptake studies. Later, integrated tests were made.

M9 medium, a chemically defined medium plus soil extract, was modified to yield a so-called "universal medium" ($\text{RM9-}^{35}\text{SO}_4$). Orthophosphate concentration was reduced to **minimal** requirement (1 mg. $\text{PO}_4\text{-P}$ per liter) to increase the detectability of change in phosphate ion concentration due to microbial activity. Sulfate with high radioactivity ($^{35}\text{SO}_4\text{-S}$) but with very little carrier ~~was~~ prepared to increase the sensitivity of the sulfate uptake. Glucose was added at a level to insure growth but to avoid its inhibitory effect on various microorganisms. Thioglycollate was included to promote better growth of anaerobes, and ~~Tris~~ buffer to maintain optimal pH.

~~Many environmental~~ factors such as aeration and growth temperature have been tested. Static growth condition and 26°C . of incubation temperature seemed to be favorable for a wide range of microbial growth. Physiologically younger cells gave higher metabolic response and a few hundred cells have been proved to be capable of initiating the metabolic activities. A number of poisons has been tested, Their effectiveness against tested organisms and soil culture insure attainability of proper poisoned control.

M9, various M9-, and $\text{RM9-}^{35}\text{SO}_4\text{-S}$ media have been tested **for** microbial metabolic activities using pure, mixed, and soil cultures, The results have been very encouraging. The possible interference ~~of~~ soil particles on metabolic activities has been studied. As the result, a method of preparing the soil inoculum has been derived, to reduce the interference to **minimum** or nil.

The engineering problems associated with mechanizing and automating a laboratory to perform the metabolic assay techniques were investigated.

The development of such an instrument appears to be feasible, although there are serious problem areas, such as the sterilization and long-term stability of reagents, which need further study. Two fundamentally different concepts - one with reusable reaction chambers and one with "throw-away" chambers were developed. Within each concept, several configurations were studied.

Although the detailed design of an instrument was outside the scope of this program, the studies show that a system employing reusable chambers and a tape-transported filter mechanism offers the most in simplicity, size, weight, and reliability,

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Automated Microbial Metabolism Laboratory
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- ✓ p. 132, line 16: change filtrates to precipitates
- ✓ p. 138, line 9: change 24 to 23
- ✓ p. 156, line 3: change 20 g.. (0.23 in. ID)
to 20 ga. (0.023 in. ID)
- ✓ p. 188, line 6: delete sentence reading "Each will
be discussed separately".
- ✓ p. 204, substitute attached revised page.



I. INTRODUCTION

The National Aeronautics and Space Administration has been planning to conduct biological experiments in manned, Earth-orbiting laboratories; on the Moon and on Mars. Important phases of these experiments will be concerned with detection of the presence of and monitoring the metabolism of microorganisms. The microorganisms may be of terrestrial, or possibly extraterrestrial origin.

In the Apollo Program, plans are being made for the astronauts to install an analytical chemistry and life detection laboratory on the Moon. It is possible that the subsurface of the Moon contains viable microorganisms, which might have come from the Earth, under the hypothesis that the Moon is of Earthly origin, or might have been deposited with dust from the Earth or from other planets or from meteorites. The metabolism portion of the lunar laboratory then should be designed to the extent possible for the detection of either terrestrial or extraterrestrial life. For the 1973 flight to Mars, an automated laboratory for life detection has been considered,

Under NASA support, Hazleton Laboratories, Inc., has been engaged in the development of an instrument to detect and monitor the metabolism of extraterrestrial life (1). This instrument supplies ^{14}C and ^{35}S labeled substrates to a soil sample and measures the production of metabolic gases. The success of this instrument has encouraged a further exploration to include other metabolic determinations and thus has lead to the present research.



Phosphate uptake and ATP production are related to the metabolism of phosphorus, which is essential to life on Earth and perhaps to life on other planets. Sulfate metabolism also is one of the vital metabolic chains for life on Earth. Many sulfate-reducing bacteria on Earth are anaerobes. They can tolerate extreme conditions of heat, pressure, salinity and cold. They resemble the organisms which can be imagined to exist in the environment of other planets.

The objective of the present contract with NASA is to probe the feasibility of detecting terrestrial life as well as extraterrestrial life through a number of "metabolic windows". More specifically, the research program is to establish feasibility parameters for monitoring microbial metabolism through uptake of phosphate and sulfate and ATP production, and to explore the engineering problems associated with the development of an integrated automated instrument to measure microbial metabolism.

The ultimate extension of the present investigations will be to combine and expand the "metabolic windows", to detect not only phosphate and sulfate uptake and ATP production, but also to incorporate the evaluation of $^{14}\text{CO}_2$ respiration as well as photosynthesis and other activities pertinent to life metabolism. All of these various metabolic activities would be detected by an integrated sensing unit which would send the information to Earth.

As stated above, the research performed at this state is to prove the experimental feasibility of monitoring phosphate and sulfate uptake and ATP production using Earth-born microbes. The engineering aspect is concerned with the concept and problems of



constructing such an integrated life detector.

II. METABOLIC UPTAKE OF PHOSPHATE

Phosphorus is essential to the metabolism of all known forms of life. Every biological reaction is ultimately dependent upon phosphorus for energy conversion and transfer (2). Furthermore, all known organisms are believed to be fastidious as to the form in which phosphorus may be accepted from the environment as orthophosphate (3). The chemical fact of the high-energy storage capacity of the resonant bonds polymerizing phosphate ions, makes phosphorus a strong candidate for a role in almost any conceivable form of extraterrestrial life.

Microorganisms take up phosphate not only at the multiplying stage, but also during the lag phase. It has been shown in a sewage study that not only did microorganisms take up phosphate during growth phases, but they even take up phosphate in the absence of growth (4). Roberts, et. al., (5) have also demonstrated rapid uptake of orthophosphate by Escherichia coli: significant amounts of ^{32}P -labeled orthophosphate were incorporated into the cells at 0°C . within three minutes.

On Earth, orthophosphate is essential to both aerobic and anaerobic metabolisms. It is reasonable to hypothesize that in the low-oxygen (or anerobic) environment on Mars, organisms would still utilize phosphate.



The experiment, then, consists of supplying a suitable aqueous medium to a sample to be tested. An aliquot of the liquid phase is removed by filtration and assayed for orthophosphate. This establishes the initial orthophosphate level contained in the medium and that added by the introduction of the sample. At periodic intervals, thereafter, aliquots are similarly removed and assayed for orthophosphate. The uptake of orthophosphate by the test culture as opposed to no uptake or attenuated uptake demonstrated in a suitably inhibited control would be evidence for metabolism.

A. Analytical Methods

Four types of analytical methods for determining the levels of orthophosphate have been studied: (1) ammonium molybdate-stannous chloride method (2) ¹⁴C-triethylamine-phosphomolybdate method, (3) enzymatic ATP assay method, and (4) calcium electrode method. Details of each experimental procedure are given and discussed below.

1. Conventional Methods

The conventional methods for the determination of phosphate suffer from at least one disadvantage as far as this program is concerned: The spectrophotometric read-out technique is not compatible with the other related experiments (e.g., Gulliver). However, the conventional methods are essential in establishing the validity of novel methods of analysis and in preliminary experiments. Therefore, unless otherwise specified, the conventional ammonium molybdate-stannous chloride method is used primarily in the phosphate uptake studies. Other conventional methods such as



ammonium molybdate-hydrazine sulfate and ammonium molybdate-aminonaphthol sulfonic acid methods are discussed.

a. Ammonium Molybdate-Stannous Chloride Method

Based on the procedure in Standard Methods for the Examination of Water and Wastewater (6), the following ammonium molybdate-stannous chloride method has been developed. This method was the primary method used for phosphate uptake studies.

Reagents :

- I. Ammonium molybdate - 10% aqueous ammonium molybdate hydrate ($.4\text{H}_2\text{O}$) is diluted 1:3 with 50% H_2SO_4 .
- II. Stannous chloride - 400 mg. of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ is dissolved in 100 ml. of 10% HCl (prepared daily).
- III. Phosphate standards - stock solution (1000 mg. phosphate-P/liter) contains 4.3916 g. of anhydrous KH_2PO_4 in 1000 ml. A few ml. of CHCl_3 are added as a preservative. Dilute solutions (down to $1\mu\text{g/ml}$) are stored in the refrigerator.

Procedure:

- I. Add 10 ml. of sample or standard to assay tube.
- II. Add 0.1 ml. of ammonium molybdate reagent and swirl.
- III. Immediately add 0.1 ml. of stannous chloride reagent and swirl.
- IV. After 12 to 15 minutes at room temperature, read vs. reagent blank in a Beckman DB Spectrophotometer 20 at 650 $\text{m}\mu$ in a 3/4 inch cell. against a reagent blank.

Alternatives :

When larger amounts of assay solution are desired, 100 ml. are placed in 250-ml. Erlenmeyer flasks and 1.0 ml. of each of subsequent reagents added. Originally, a water blank was used instead of a reagent blank, and a correction factor had to be applied to each reading.



Results : A typical response curve is given in Figure No. 1, and indicates reasonably good sensitivity down to about 25 $\mu\text{g.}$ of phosphate-P per liter. Sensitivity could be extended by the use of a longer light path.

b. Ammonium Molybdate-Hydrazine Sulfate Method

Based on the procedure of Bruice, ~~et al.~~, (21), the following method was tested.

- Reagents:
- I. 5 N H_2SO_4
 - II. 0.15% hydrazine sulfate (w/v, distilled water)
 - III. 2.5% ammonium molybdate (.4 H_2O) (w/v, distilled water)

- Procedure :
- I. To 5 ml. of sample in 10-ml. volumetric flask, add 2 ml. of 5 N H_2SO_4 .
 - II. Add 0.5 ml. of hydrazine sulfate reagent and 1.0 ml. of the ammonium molybdate reagent.
 - III. After filling to volume with distilled water and mixing, transfer to tube. Boil for 10 minutes, cool and read in 4 cm. cell in Beckman DB at 730 $\text{m}\mu$ against a reagent blank.

Results : A typical curve is included in Figure No. 2 and indicates reasonably good sensitivity even at 5 $\mu\text{g.}$ phosphate-P per liter.

c. Ammonium Molybdate-Aminonaphthol Sulfonic Acid Method

Based on the procedure of Sunderman and Sunderman (7), the following method was tested:

- Reagents:
- I. 5 N H_2SO_4
 - II. 2.5% ammonium molybdate .4 H_2O (w/v) in distilled water.
 - III. Aminonaphthol sulfonic acid reagent:
To 19.5 ml. of 15% sodium bisulfite (w/v)

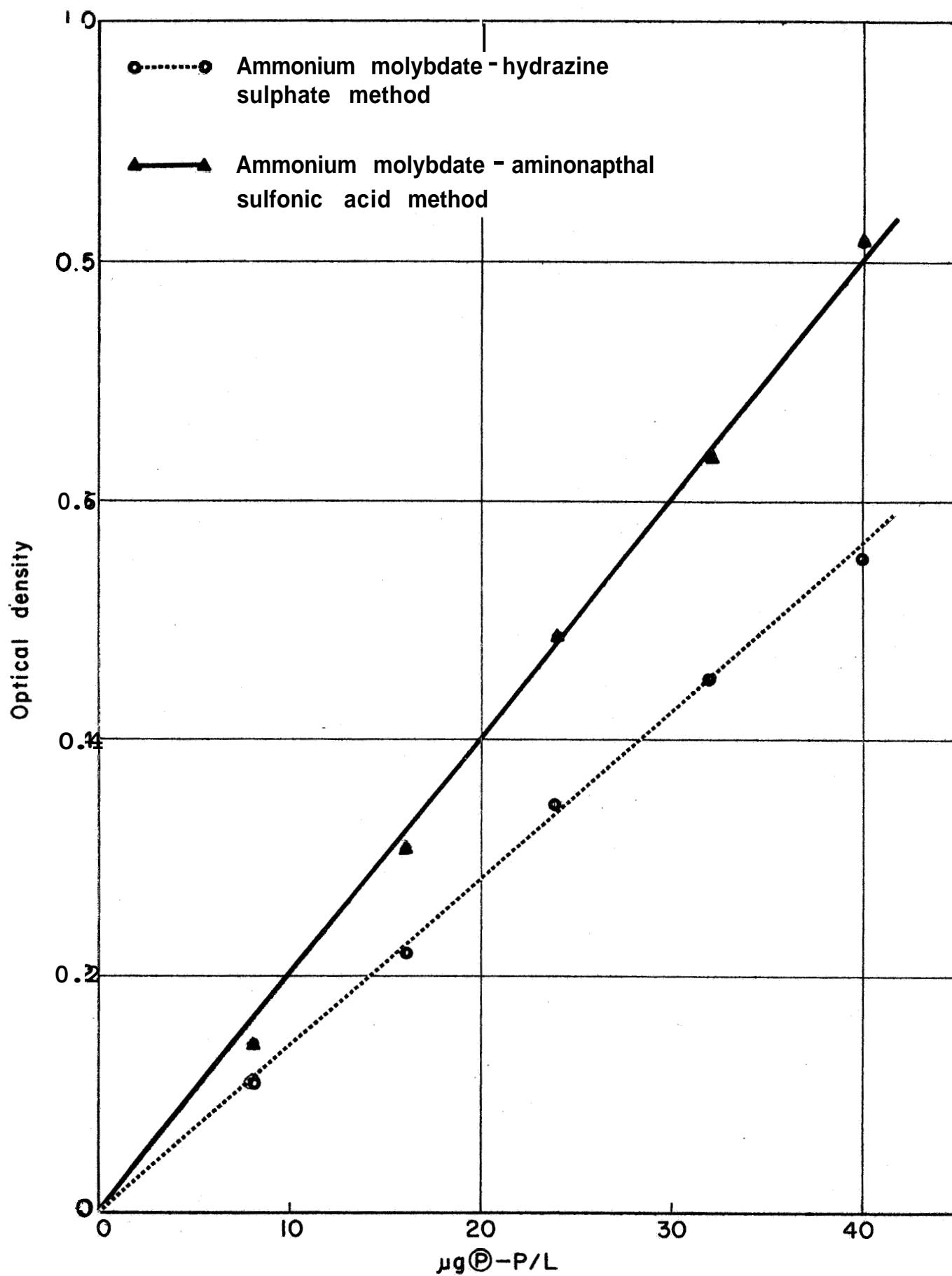


Figure No. I —Phosphate—P standard curves by two conventional methods.

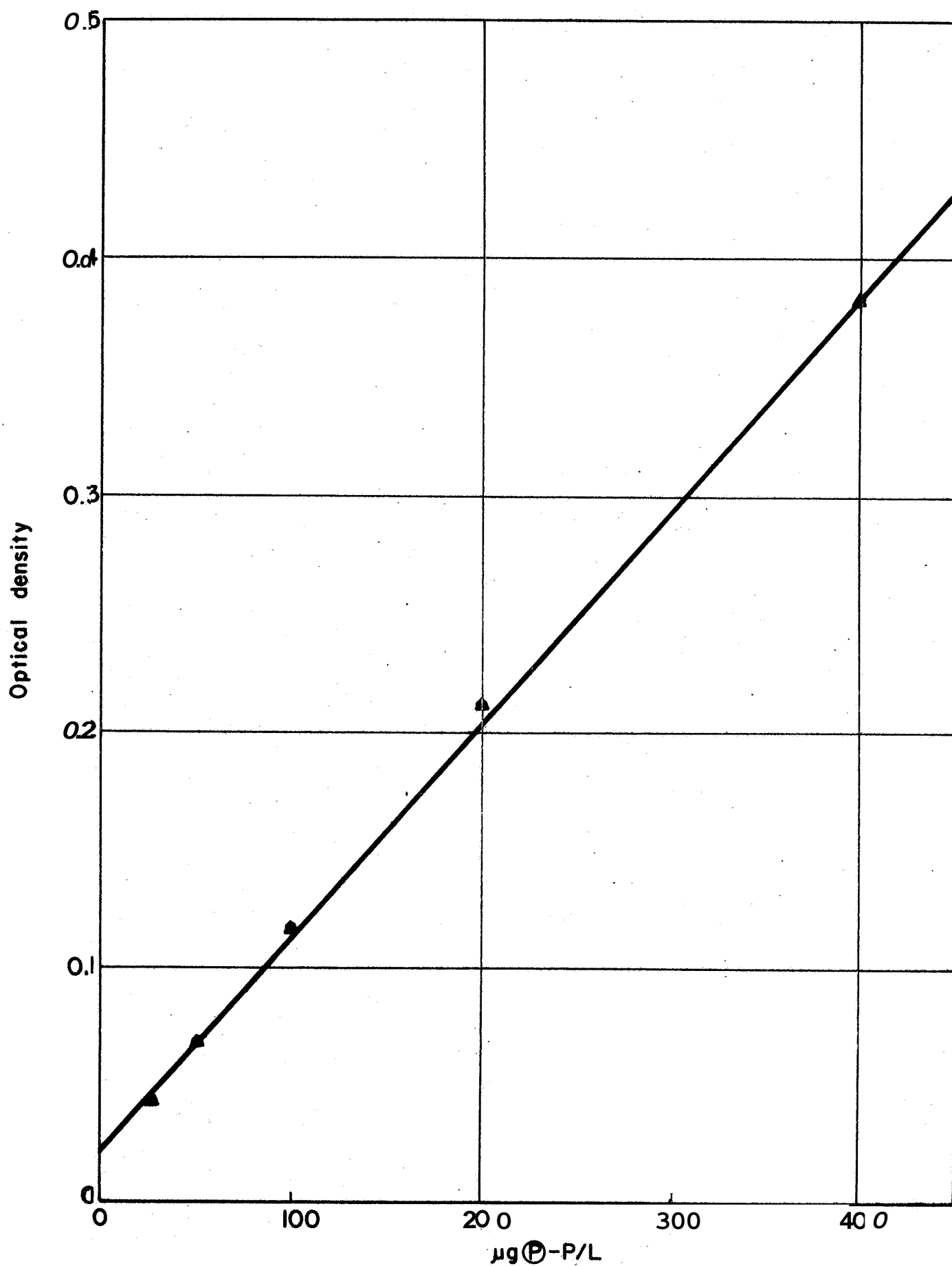


Figure No.2— Phosphate -P standard curve, ammonium molybdate-stannous chloride method.



in a glass-stoppered cylinder are added 0.5 g. of 1,2,4-aminonaphthol sulfonic acid. After adding five ml. of 20% sodium sulfite (w/v), the flask is shaken until the sulfonic acid goes into solution. (The reagent is stable for one month in the refrigerator).

Procedure:

- I. To 15 ml. of sample or standard in a 25-ml. volumetric flask are added, successively, 2.5 ml. of 5 N H_2SO_4 , 2.5 ml. of ammonium molybdate solution and 1.0 ml. of the aminonaphthol sulfonic acid reagent.
- II. After filling to volume with distilled water, the mixture is allowed to stand for five minutes at room temperature and then read against a reagent blank, in a 4 cm. cell in the Beckman DB Spectrophotometer, at 700 m μ .

Results :

A typical curve is included in Figure No. 2 and shown sensitivity of about 8 μ g. phosphate-P per liter.

It should be noted that the first described method was measured in one cm. cell, whereas the latter two methods were measured in four cm. cells. Nevertheless, the third method is more sensitive than the second which is more sensitive than the first, even after multiplying the O.D. readings of the first method by four.

2 ^{14}C -triethylamine-phosphomolybdate Method

The obvious method for radioactive assay of orthophosphate is to use the isotope ^{32}P . However, the relatively short half-life (14.3 days) precludes the use of this isotope for a Mars mission. However, it should be possible to utilize the long-lived isotope, ^{14}C , by quantitatively precipitating phosphate with ^{14}C -triethylamine hydrochloride in the Sugino-Miyoshi procedure.

Sugino and Miyoshi (8), have described a variation of the ammonium molybdate method of determining the concentration of orthophosphate which depends on the formation of a phosphate-triethylamine-



molybdate precipitate rather than the formation of a colored complex, as in the above more conventional methods. Not only is this method not sufficiently sensitive (it is good to 5×10^{-6} M phosphate), but it would not be practicable in this program. However, there is the possibility that substitution of ^{14}C -labeled triethylamine for the unlabeled material **may** yield a technique which is sufficiently sensitive to measure phosphate uptake by a few microorganisms and which would have a compatible read-out with the other experiments in the program.

- Reagents :
- I. 4 N perchloric acid
 - II. 0.08 M ammonium molybdate
 - III. 0.8 M triethylamine hydrochloride

- Procedure :
- I. To 1.0 ml. of sample or standard, add successively 0.05 ml. of 4 N perchloric acid, 0.25 ml. of 0.08 M ammonium molybdate and 0.05 ml. of triethylamine hydrochloride.
 - II. After a few minutes at room temperature, the precipitate is removed by centrifuging at 1500 x g. for five minutes.
 - III. The concentration of phosphate in the supernatant is determined by one of the conventional methods; the total amount of phosphate in the precipitate is determined by dissolving the precipitate in 2 ml. of 1 N ammonia, then diluting 1:500 with water and using a conventional assay to determine phosphate recovery.

Results : Typical results are summarized in Table No. 1. As can be seen, the recovery of phosphate by triethylamine-ammonium molybdate-perchloric acid mixture **was** very good. A high specific activity of triethylamine used to recover phosphate would be expected to yield the most sensitive results.

Following are the tests using ^{14}C -triethylamine

- Reagents :
- I. 4 N perchloric acid

Table No. 1 - Phosphate recovery by precipitation method

<u>CONC. OF PHOSPHATE IN STARTING SOLN.</u>	<u>RECOVERY OF PHOSPHATE</u>
<u>mg/L</u>	<u>mg/L</u>
0.10	0.08
0.20	0.21
0.40	0.42
0.60	0.60
0.80	0.76



11. 0.08 M ammonium molybdate

111. ^{14}C -triethylamine

As received, the triethylamine had a specific activity of 1.1 millicuries per millimole. There were 7.8 millicuries per ml. and 7.1 millimoles per ml. The contents were diluted 1:100 with water to elute them. This solution was stored in the refrigerator; it contained $78 \mu\text{C}/\text{ml}$ or $1.7 \times 10^8 \text{ DPM}$.

- Procedure:
- I. The ^{14}C -triethylamine solution was further diluted 1:10 with 0.8 M triethylamine hydrochloride (unlabeled). To 1.0 ml. of varying concentrations of phosphate were added 0.05 ml. of 4 N perchloric acid, 0.25 ml. of ammonium molybdate and 0.05 ml. of the labeled triethylamine hydrochloride,
 11. After a few minutes at room temperature, the tube was centrifuged at $1500 \times g$ for five minutes.
 111. The supernatant was placed in a planchet and 1 ml. of solution containing ammonium molybdate and perchloric acid was added to the centrifuge tube.
 - IV. After resuspending the precipitate, it was centrifuged again. The supernatant was added to the original planchet and the precipitate to a second planchet. After evaporating the solutions in the planchets to dryness, they were counted in a Widebeta gas proportional counter (with an efficiency of approximately 30%).

Results: Typical results for the precipitate are shown in Figure No. 3. The limits of sensitivity are difficult to determine on this curve, but a linear curve of cpm vs. standard phosphate concentration ranging from 0 to $100 \mu\text{g PO}_4\text{-P}$ per liter was established.

Although this method appears promising, the recovery of phosphate molybdate precipitate was made by centrifugation, which obviously is not applicable for flight instrument. Therefore, an approach of using membrane filtration for recovery of the precipitate was attempted,

- Procedure:
1. The ^{14}C -triethylamine stock solution (1:100 dilution) was further diluted to 1:10 with 0.8 M triethylamine hydrochloride (unlabeled). To 2.0 ml. of varying concentration of phosphate were added 0.1 ml. of 4 N perchloric acid, 0.5 ml. of 0.08 M ammonium molybdate, and 0.1 ml. of ^{14}C -triethylamine. A standard curve was made, this time at the range of 10 pg through $1000 \mu\text{g}$ per liter to cover a wider range.

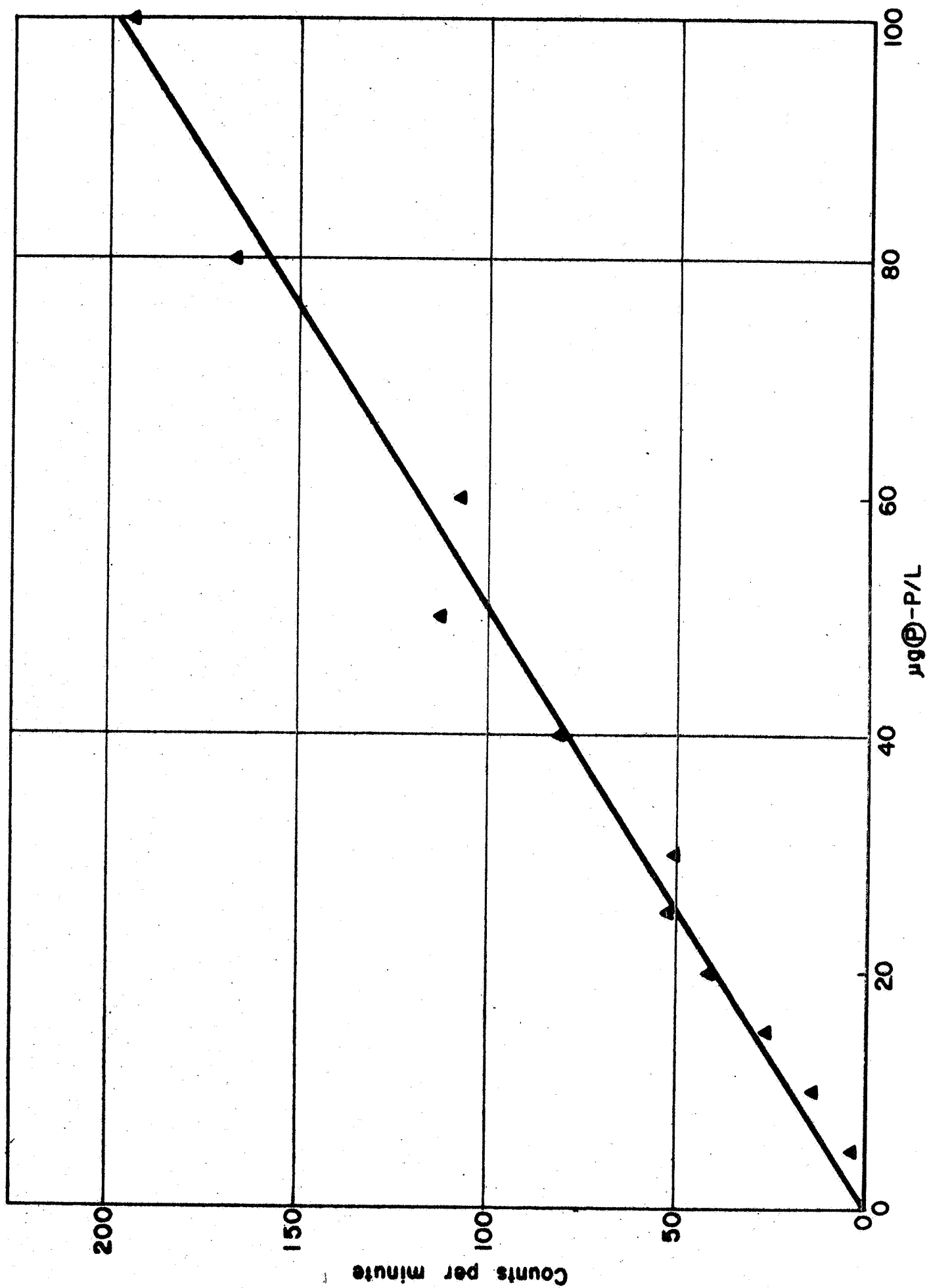


Figure No 3 - Phosphate - P standard curve using ^{14}C -triethylamine



Results :

The results are shown in Figure No. 4. Although the cpm was low, the linearity of the curve was encouraging.

The radioactivities of the precipitates shown in Figures No. 3 and No. 4 are rather low. The highest value was less than 200 cpm which is only approximately 10 times higher than the background count using Nuclear Chicago D-47 gas flow counter. To obtain more highly radioactive precipitates, it is necessary either to use higher specific activity or larger amounts of ^{14}C -triethylamine material. Since the available ^{14}C -triethylamine was low in activity, larger amounts of ^{14}C -triethylamine were used in preparing the high radioactive precipitate.

The new batch of ^{14}C -triethylamine had a specific activity of 0.59 millicurie per millimole. The material (1.7 mg.) was dissolved in 0.63 ml. distilled water and then subsequently diluted 1:50 with 0.8 M unlabeled triethylamine for preparing the working solution. One-tenth, 0.25, 0.5, 0.75 or 1.0 ml. of this working solution plus 0.1 ml of 4 N perchloric acid, 0.5 ml. of 0.08 M ammonium molybdate were added to one ml. of RM9 medium containing 1000 μg . $\text{PO}_4\text{-P}$ per liter. The precipitates, after being washed, showed radioactivities ranging from 900 cpm to 5700 cpm, as presented in Figure No. 5. The highest cpm was obtained by using 0.5 ml. of working ^{14}C -triethylamine solution. Further increase of this solution decreases the cpm of the precipitates.

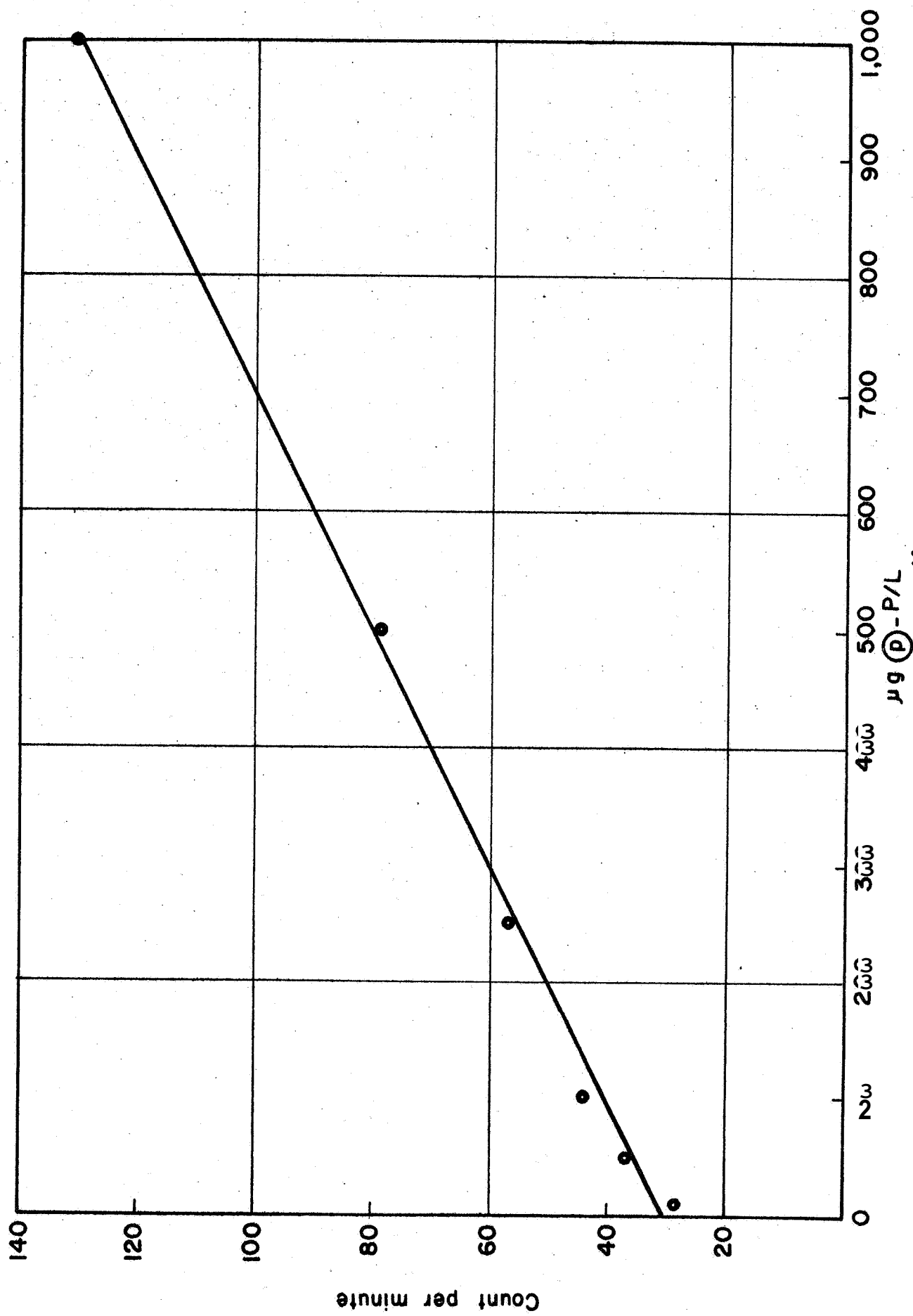


Figure No.4 — Phosphate — μ stondord cul-e using ^{14}C - triethylamine

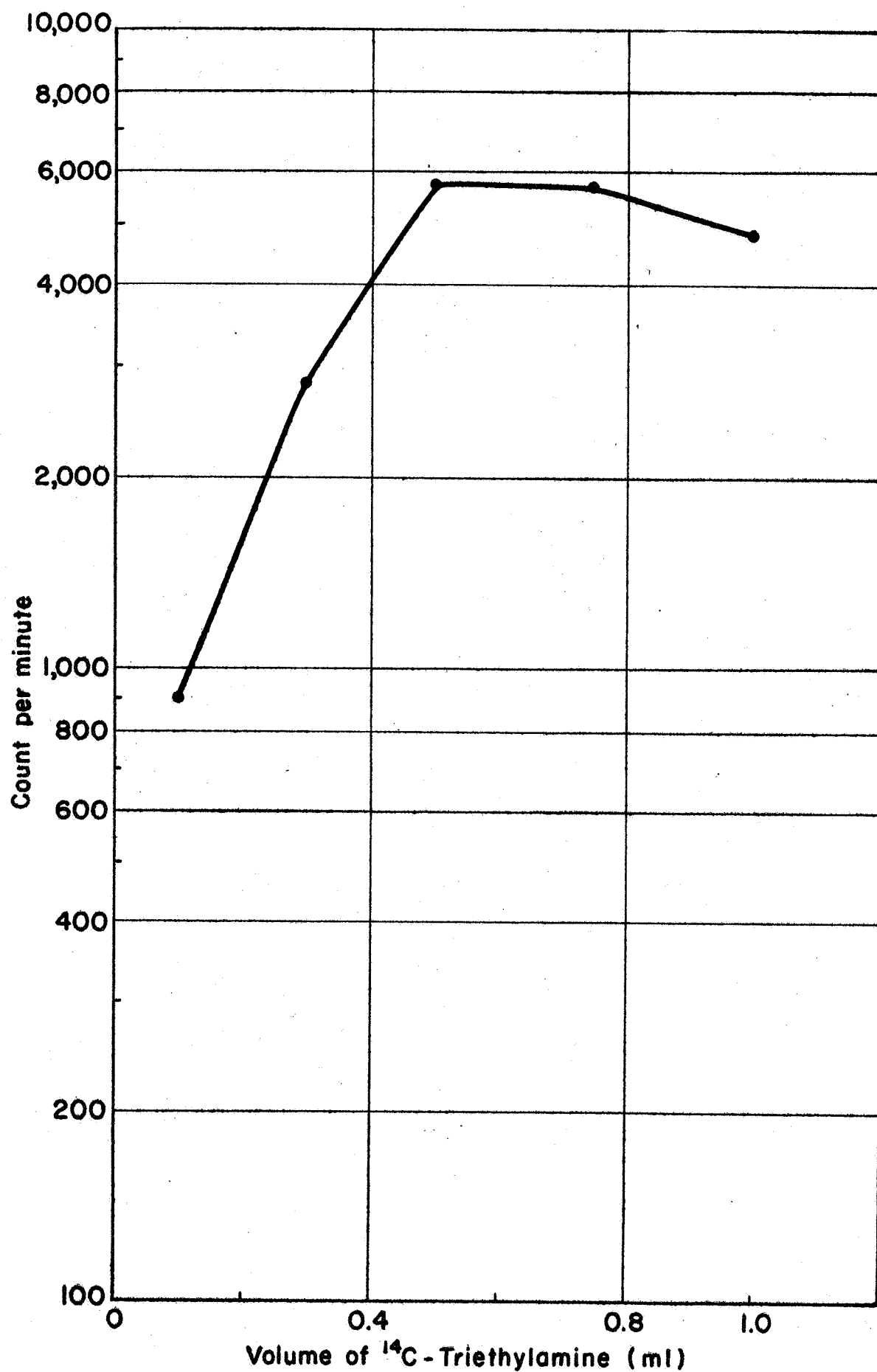


Figure No.5 - Radioactivity count of ^{14}C -Triethylamine - Phosphomolybdate precipitated by various concentrations of ^{14}C -Triethylamine



3. Calcium Electrode Method

Another method of measuring phosphate which would be compatible with the other elements in the Gulliver package would be with a specific ion electrode. A phosphate ion electrode is not available; however, a calcium ion electrode Model 92-20 (Orion) was procured. This electrode is sensitive to concentrations of calcium ion from saturated solutions down to 10^{-4} M (1.2×10^{-6} g. per liter). Attempts have been made to use this electrode to measure concentrations of phosphate indirectly. This indirect and simple method to determine orthophosphate uptake is based on the measurement of calcium ion released from a relatively insoluble phosphate such as $\text{Ca}_3(\text{PO}_4)_2$ as dissolved phosphate is metabolically taken up from a saturated solution. By comparing the calcium ion at zero-time incubation and after an interval of incubation, the increased calcium ion should represent an equivalent amount of orthophosphate being taken up by microorganisms.

Standard curves of calcium ion vs. millivolts are shown in Figure No. 6. Various concentrations of calcium ion in distilled water, M9 and M11 media were tested. The resulting curves were parallel and nearly identical. However, attempts to correlate calcium ion change with growth of *E. coli* in M9 plus $\text{Ca}_3(\text{PO}_4)_2$, CaHPO_4 and $\text{Ca}(\text{H}_2\text{PO}_4)_2$ were not very successful.

4. Enzymatic Method

A number of attempts have been made to utilize the conversion of inorganic phosphate to ATP as a basis for determining the amount of phosphate present in the medium before and after incubation with microorganisms. All of the substrate and enzymes required for the

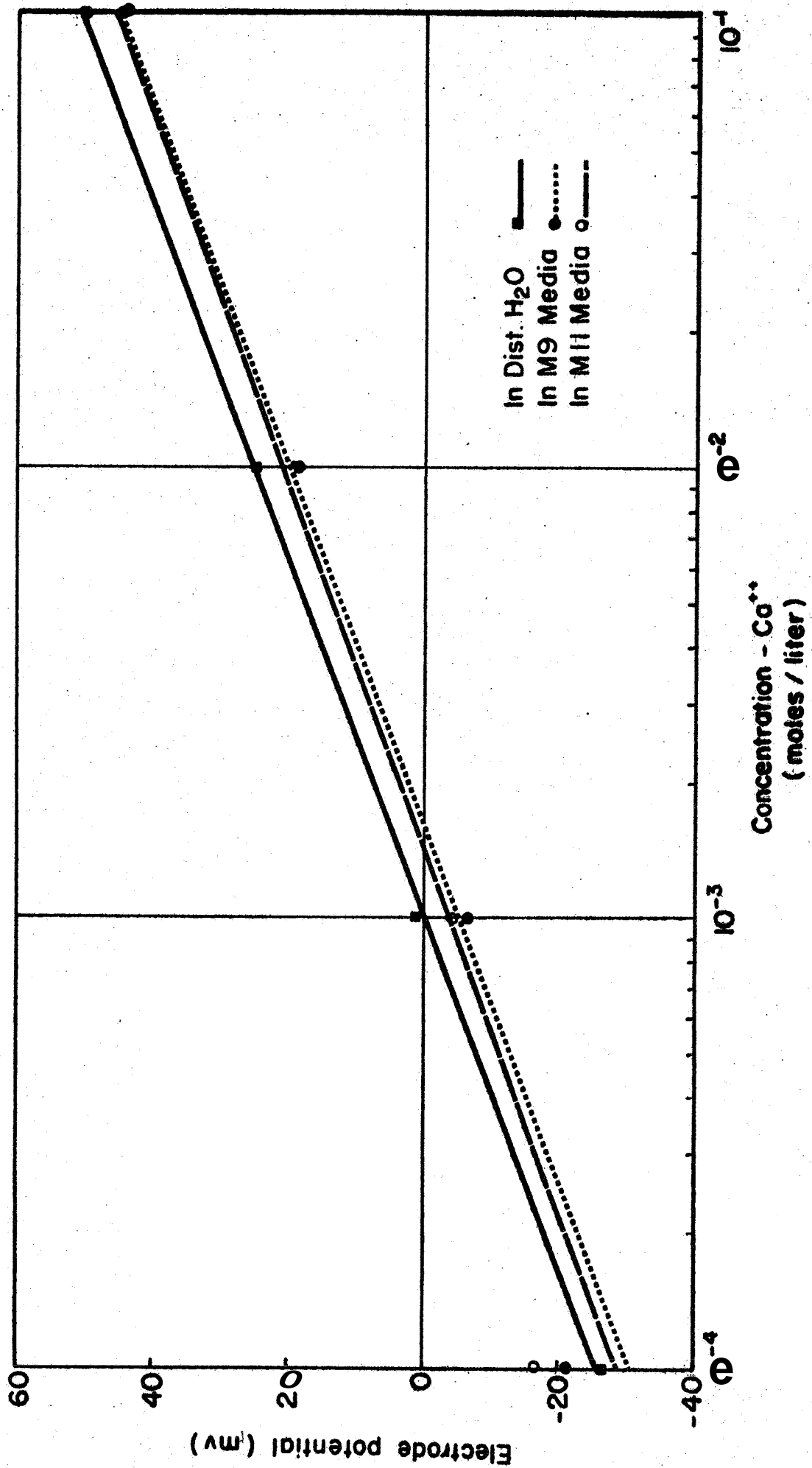
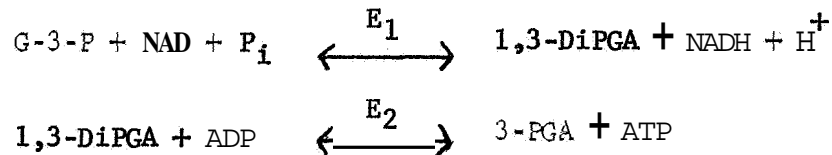


Figure No.6 - Standard curve - calcium concentration vs. electrode potential



following reaction sequences have been obtained;



where: G-3-P is glyceraldehyde-3-phosphate
NAD is nicotinamide adenine dinucleotide
 P_i is inorganic orthophosphate
 E_1 is phosphoglyceraldehyde dehydrogenase
1,3-DiPGA is 1,3-diphosphoglyceric acid
NADH is reduced NAD
 E_2 is 3-phosphoglyceric acid kinase
3-PGA is 3-phosphoglyceric acid,

The ATP read-out is via the firefly luciferin-luciferase system. As performed in our laboratory, this system was able to detect as little as 10^{-7} $\mu\text{g.}$ of ATP. Much work has been done on the conversion of P_i to ATP. However, the results have not been very promising. The main reason for the unsuccessful attempts is due to contamination by inorganic phosphate in many of the reactants shown in the above equations. For example, the inorganic phosphate in G-3-P was 30 $\mu\text{g.}$ per ml; ADP, 0.60 $\mu\text{g.}$ per ml. and 3-phosphoglyceric acid kinase, 2 $\mu\text{g.}$ per ml. In order to make this detection system useful for determining inorganic phosphate, the sensitivity of the assay range should be less than 1 ppm since the RM9 medium (See Table No.3) contains only 1 mg. per liter of $\text{PO}_4\text{-P.}$ Phosphate contamination in the reactants discussed above is a drawback of the enzymatic inorganic phosphate determination method.



In addition to the problem of $\text{PO}_4\text{-P}$ contamination, the ADP component also **was** contaminated with ATP compound. A paper chromatograph method has **demonstrated** that the ADP of Sigma Chemicals **Co.**, contained at least **two** compounds. The contaminant had R_x value close to ATP.

Attempts to establish a dose-response curve, using various concentrations of inorganic phosphate (up to 10 μg . per ml. $\text{PO}_4\text{-P}$) yielded only a single value indicating that the range of inorganic phosphate **was** below the concentration of the exogenous contamination from the reactants.

B. Experimental

1. Development of Medium

a. General Aspects

As in the Gulliver project, **it** is necessary to use an almost "universal" growth medium, i.e., a medium in which almost all microorganisms will grow to some extent. Obviously, this "universal" growth medium **will** not be optimal for all, or most, or even possibly any of the organisms. As a starting point, **some** experiments were done using media M9 and M11 developed previously (see Tables No. 2 and No. 3). These media are not well suited for phosphate uptake experiments since they each contain high amounts of phosphate (1.0 g/L). It is a well-established principle that a **small** difference between two large numbers is more difficult to detect than that **same** difference between two **small** numbers. Thus, to detect the phosphate uptake of a few microorganisms, **it** will be necessary to have a medium with a low phosphate concentration. **It** will, of course, have to be

Table No. 2 - Composition of M9.

K_2HPO_4	1.0 g/L
NH_4NO_3	0.2 g/L
$MgSO_4 \cdot 7H_2O$	0.2 g/L
NaCl	0.1 g/L
Soil Extract*	100.0 ml.

pH 7.0

*

Soil extract prepared by suspending 500 g. of air-dried soil in 1300 ml. H_2O . Mixture is autoclaved for one hour, filtered, and liquid loss made up to 1000 ml. with distilled sterile water.

Table No. 3 - Composition of M11.

K_2HPO_4	1.0 g/L
KNO_3	0.031 g/L
$MgSO_4 \cdot 7H_2O$	0.2 g/L
NaCl	0.1 g/L
Malt Extract	0.19 g/L
Beef Extract	0.19 g/L
Yeast Extract	0.81 g/L
Ascorbic Acid	0.013 g/L
L-cystine	0.044 g/L
Bacto-casamino Acid	0.25 g/L
Proteose peptone #3	1.25 g/L
soil Extract*	16.0 ml/L
NH_4NO_3	0.19 g/L

*

Same treatment as for M9.

demonstrated that the low phosphate medium will sustain metabolism sufficiently for the experiment. As a first step, Media M9 and M11 were used, merely decreasing the concentrations of phosphate and adding glucose. The latter was added as a carbohydrate source to promote additional phosphate uptake through substrate phosphorylation. Since growth of E. coli was limited on such a medium, later experiments have depended on the technique of growing the organisms on a more optimal medium (Difco Lactose Broth), depleting phosphate reserves by placing the cells in a phosphate-free solution, and then transferring the cells to phosphate-low M11 or M9 for the uptake experiments. Obviously, it is not proposed to use this system in the final process, but it was convenient in the preliminary experiments which were required in order to establish the most sensitive assay system.

The M11 medium was rejected for the sulfate uptake study because the medium contained L-cystine which is strong competition for $^{35}\text{SO}_4\text{-S}$ in microbial metabolism. The L-cystine is in a reduced form, while the $^{35}\text{SO}_4\text{-S}$ is in the oxidized form. As a general rule, microorganisms preferably take up the reduced form rather than the oxidized form of sulfur compound.

Further development on a "universal medium" was then concentrated and conducted on M9 medium. This medium, as can be seen in Table No. 2, is a rather simple medium. In addition to K_2HPO_4 , NH_4NO_3 , MgSO_4 , and NaCl the soil extract provides trace elements and perhaps vitamins and other organic substances which are vital to many microbial growths. The glucose concentration as stated above is important to phosphate uptake, but at higher concentrations



many microorganisms, especially some autotrophs, could be suppressed.

The final version of the so called "universal medium" was derived from M9 medium. This medium is now called RM9 (revised M9 medium). The compositions of both RM9 and M9 are listed in Table No. 4 for comparison.

The concentrations of three components which were originally in M9 were changed to increase the detection sensitivity for microbial activities. Potassium phosphate was reduced from 1.0 gram to 5.0 mg. (or 1.0 mg. $\text{PO}_4\text{-P}$) per liter and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, from 0.2 g. to 0.08 g. per liter. The phosphate and sulfate concentrations at the reduced levels did not affect the growth as compared with the M9 medium, but the sensitivity of detecting the change of these compounds due to microbial activities has significantly increased. Theoretical consideration and the experimental basis for selecting 5.0 mg. per liter for K_2HPO_4 and 0.08 g. per liter for $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ will be discussed later,

Glucose concentration was established at the 0.2 g. per liter level for the following reason: The carbon content of bacteria on the average is 50%. Assuming a medium can support the growth of bacteria, E. coli, for example, up to 10^7 cells per ml. , the total bacterial mass will be 6.7 μg . The dry weight of a single E. coli is 6.7×10^{-13} g. (9). Therefore, the carbon content of 10^7 cells will be 3.42 μg . The heterotrophic organisms require approximately 10 parts of carbon to synthesize one part of water-free substance, therefore, 34 μg . ($3.4 \times 10 \mu\text{g}$.) of carbon are required to produce 10^7 cells. Since the carbon content in

Table No. 4 - Composition of revised M9 (RM9)
and original M9 media.

R (REVISED) M9			ORIGINAL M9		
K_2HPO_4	5	mg.	K_2HPO_4	1.00	g.
$MgSO_4 \cdot 7H_2O$	0.08	g.	$MgSO_4 \cdot 7H_2O$	0.20	g.
NH_4NO_3	0.20	g.	NH_4NO_3	0.20	g.
NaCl	0.10	g.	NaCl	0.10	g.
Soil Extract	100	ml.	Soil Extract	100	ml.
Glucose	0.2	g.	Glucose	0.005	g.
Na-thioglycollate*	0.002	g.	Distilled Water	900	ml.
Tris	6	g.	pH 7.0		
Distilled Water	900	ml.			
pH 7.0					

* Solution of 20 mg. Na-thioglycollate in 10 ml. distilled water was prepared each week. It was added to the medium after being heated (by a steam bath) for 10 minutes.



glucose is 40%, to synthesize 10^7 cells per ml. medium, at least 0.085 mg. glucose per ml. is required. When RM9 contains 0.2 g. glucose per liter, the glucose content is sufficient to support the growth of at least 10^7 cells per ml. or more. This calculation does not include organic materials from soil extract, therefore, the microbial growth in RM9 should be expected to reach around 10^8 cells per ml. Under such a low concentration of glucose, the probability that the medium can support both heterotrophic and facultative autotrophic growth, or possibly strict autotrophic growth, is great.

Two additional components have been incorporated in the new M9 medium. These are Na-thioglycollate and Tris. The former is to reduce the oxidation-reduction potential in the medium to facilitate the growth of anaerobes. At the levels of 2.0 to 10 mg. per liter, the thioglycollate does not affect adversely the growth of a strict aerobic organism. If required, the amount may be further increased. The Tris was added to increase the buffer capacity of the medium, since the phosphate content in RM9 has been substantially reduced from 1.0 gram to 5.0 mg. per liter. The Tris will be able to maintain the pH at a rather stable value.

b. Phosphate Concentration in the Test Medium

The RM9 medium contains 1 mg. $\text{PO}_4\text{-P}$ per liter. The experimental data supporting this change are given below:

In the preliminary experiment, M9 medium with 0.5% glucose supplement was inoculated with E. coli and incubated at 37°C . with duplicate flasks both in shaking and in stationary conditions. Aliquots were removed at 24 hours, filtered through a Gelman GA-6 metricel

filter (0.45 μ .) to remove bacteria, and after suitable dilution, the phosphate level was determined on the filtrate. The phosphate assays were made by the conventional ammonium molybdate-stannous chloride method, and by non-radioactive triethylamine precipitation method. In the latter case, both the precipitate and the supernatant remaining after precipitation were assayed for phosphate level (by the ammonium molybdate-stannous chloride method). The results are summarized in Table No. 5. The completeness of precipitation in the triethylamine ammonium molybdate procedure can be seen here.

To increase the sensitivity of measuring phosphate uptake, the phosphate content of the media was decreased by decreasing the amount of K_2HPO_4 from 1.0 g/L to 0.2g/L. Using this decreased concentration of phosphate (0.2 g. K_2HPO_4 per liter of medium), the concentration of phosphate not assimilated in E. coli cells was measured by the ammonium molybdate-stannous chloride procedure for several time periods of incubation. The results are summarized in Table No. 6. In all cases, there is a dramatic decrease in the concentration of phosphate, indicating uptake by the cells, followed by the unexplained return toward the original phosphate levels noted in Reference 4.

Cultures of E. coli and Bacillus subtilis, var. globigii (BG), were grown for 24 hours; the ~~E. coli~~ had approximately 8×10^{10} cells/ml. and the BG, 4×10^{10} cells/ml., based on O.D. After washing twice with physiological saline, the cultures were suspended in saline and placed in a refrigerator for two hours. Duplicate 0.5 ml. aliquots of ~~E. coli~~ and 0.25 ml. aliquots of BG were placed in flasks containing 25 ml. of M9 medium supplemented with 0.8% glucose and with K_2HPO_4 decreased to 10 mg/L. The E. coli gave a plate count at this point of 1×10^8

Table No. 5 - Phosphate assay by colorimetric and precipitation methods*.

	COLORIMETRIC METHOD	PRECIPITATION METHOD**	
		PRECIPITATE	SUPERNATANT
Sterile Control	217.5 mg/L	220.6 mg/L	0.6 mg/L
<u>E. coli</u> , shaken, 37°, 24 hours	207.5	249.9	0.4
<u>E. coli</u> , stationary, 37°, 24 hours	232.5#	238.1	0.5

*

Medium is M9 with a 0.5% glucose supplement.

** The precipitation method results are expressed in **terms** of original solution - i.e., the precipitate is dissolved in aqueous ammonia and then brought back to the original volume.

This value must represent phosphate contained in the inoculum which was released during incubation.

Table No. 6 - Phosphate uptake by E. coli
for various periods of incu-
bation at 37°.

<u>MEDIUM* AND MODE</u>	<u>PO₄-P IN MG. PER LITER</u>			
	<u>HOURS OF INCUBATION</u>			
	<u>0</u>	<u>3</u>	<u>5</u>	<u>24</u>
M9, shaking	50	40	25	40
M9, stationary	40	5	40	44
M11, shaking	60	15	47	57
M11, stationary	90	10	50	75

*

Note: Both media had decreased K₂HPO₄ (0.2 g/L instead of 1.0 g/L)
and had 0.2% glucose added as supplement.



cells/ml. , and the BG, 1.2×10^8 cells/ml.

Samples of membrane-filtered medium were assayed at the times indicated in Table No. 7 by the ammonium molybdate-stannous chloride method. Under these conditions, as shown in the Table, there was very little phosphate uptake in the first five hours, but appreciable uptake occurred in the next 19 hours.

A similar experiment was run next, but three levels of phosphate were tested simultaneously. The respective media assayed at 1.15, 4.45, and 12.30 mg. $\text{PO}_4\text{-P}$ per liter at 0 time; these values were checked at 3, 5, 24 and 48 hours and varied only a few percent. The results obtained with *E. coli* are shown in Figure No. 7 and those with BG in Figure No. 8. The results are qualitatively very similar: at the lowest phosphate concentration (1.15 mg. of $\text{PO}_4\text{-P}$ per liter), there is very little uptake in the first five hours, but in the next 19 hours almost all of the phosphate was removed from the medium by the microorganisms. At the intermediate value in this series (4.45 mg. of $\text{PO}_4\text{-P}$ per liter), phosphate uptake can be noted even in the first three hours. Another contrast with the results obtained with the lowest phosphate medium is the release of phosphate from the organisms back into solution in the period between 24 and 48 hours. At the high phosphate concentration (12.30 mg. of $\text{PO}_4\text{-P}$ per liter), the results were similar to those obtained with the intermediate level, except that both the rate of uptake of phosphate in the early period and the rate of phosphate release in the late period were enhanced. (The results given in Figure No. 7 and No. 8 were obtained with 1.5×10^9 cells/ml. in each case).

Table No. 7 - Phosphate uptake in M9 medium
with 10 mg/L K_2HPO_4 and 0.2%
glucose; ca. 10^8 cells/ml.

SYSTEM	PO ₄ -P IN MG. PER LITER			
	HOURS OF INCUBATION			
	<u>0</u>	<u>3</u>	<u>5</u>	<u>24</u>
Control	2.15	2.14	2.10	2.10
<u>E. coli</u> , 1	2.00	2.21	2.00	1.35
<u>E. coli</u> , 2	2.10	2.02	1.90	1.15
BG, 1	2.08	2.10	2.10	1.20
BG, 2	2.12	2.15	2.02	1.40

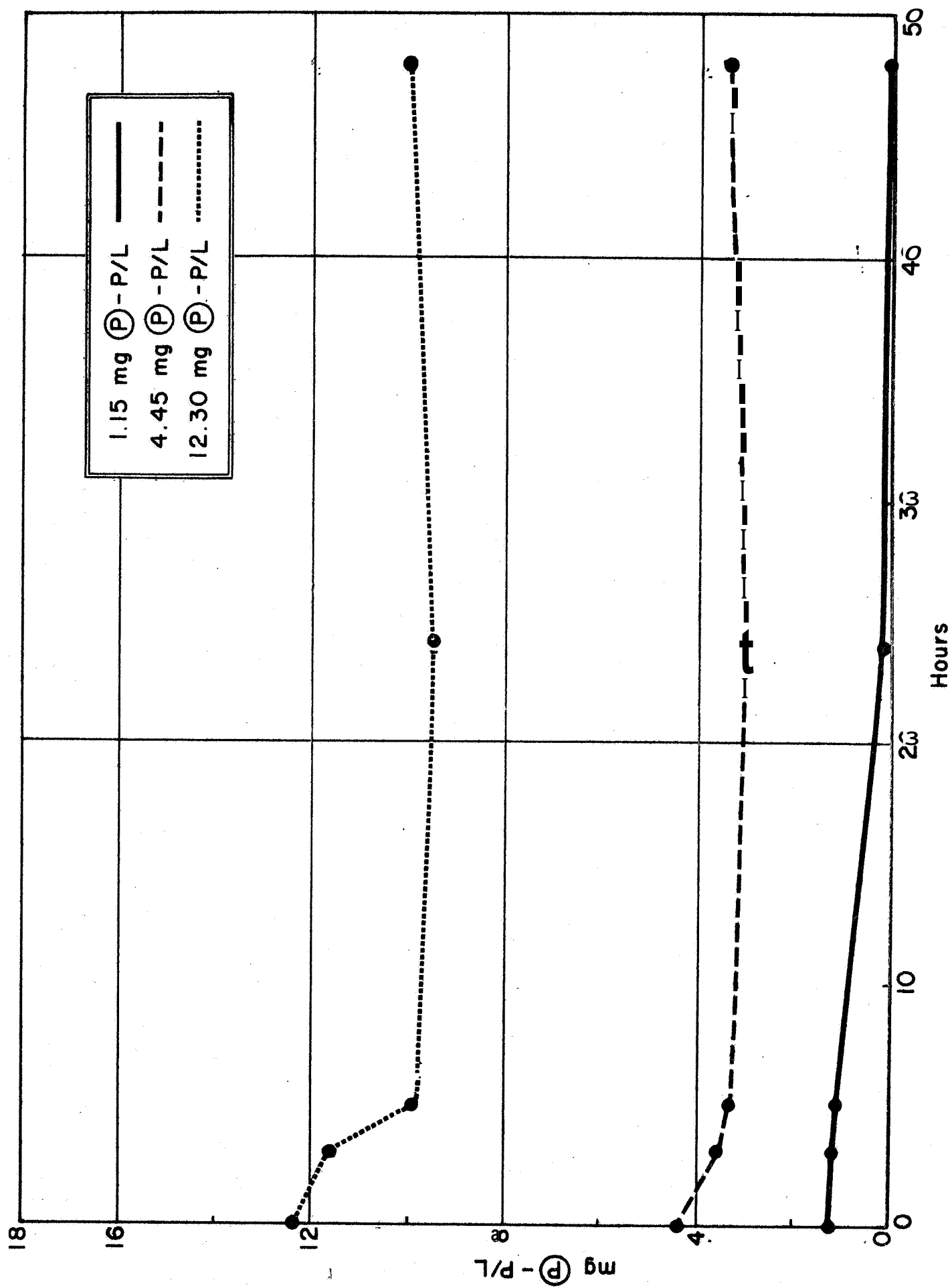


Figure No. 7 — Phosphate uptake by *E. coli*, various concentrations of phosphate in medium.

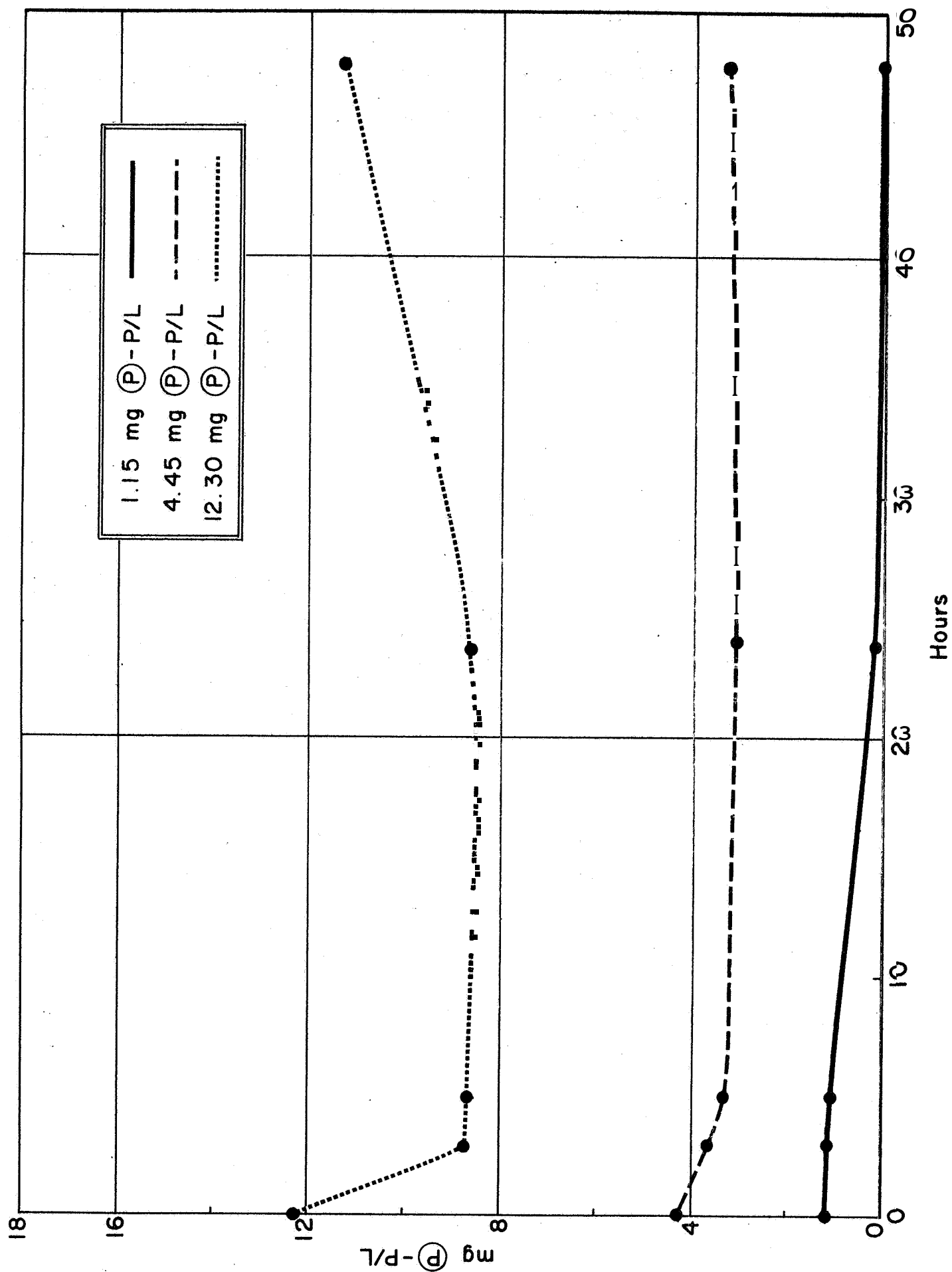


Figure No 8 - Phosphate uptake by BG, various concentrations of phosphate in medium.



Because the phosphate uptake **was** nearly complete in 20 to 30 hours, when the microorganisms were growing in the low phosphate concentration (1 mg. per liter) and no phosphate leaching occurred with further incubation (as did occur when the higher phosphate concentrations of 4 or 12 mg. $\text{PO}_4\text{-P}$ per liter were present in the medium) the lower phosphate concentration was chosen to be the concentration in the test medium.

2. Effect of Selected Poisons on Microbial Growth And Phosphate Analysis

The ideal poisons selected for the present program should have the following features: (1) preferably a germicidal rather than a germ static toward microbial cells; (2) **no** interference with phosphate analysis; (3) no leaching of phosphate from cells; (4) no interaction with the medium ingredients to form precipitates and (5) preferably non-volatile compounds. Although the Bard-Parker germicide (patented germicide produced **by** Bard-Parker Co., Inc., Danbury, Connecticut) is very effective, **some** of its ingredients are volatile.

So far the poisons which have been used for phosphate uptake studies and proved to be effective are:

- (1) Bard-Parker (0.3 ml. per 40 ml. media)
- (2) 0.66 mM 2,4-dinitrophenol
- (3) Seventy percent perchloric acid
(0.3 - 0.6 ml. per 40 ml.)

Other poisons have also been studied, such as toluene, phenol and 4% Ceepryn, Table No. 8 shows the result of 4% Ceepryn and 70% perchloric acid on inhibition of *E. coli* and *P. fluorescens* growth. Although the Ceepryn is a very strong germicide, **it** is not suitable to be used as a poison for poison control samples because

Table No. 8 - Effect of poisons on microbial growth and phosphate analysis.

CULTURES	SYSTEMS ML. POISON IN 40 ML. RM9	0 HR.		24 HR.	
		CELL DENSITY (NO. PER ML.)	PO ₄ -P (MG/L)	CELL DENSITY (NO. PER ML.)	PO ₄ -P (MG/L)
<u>E. coli</u>	Perchloric acid	0			
		0.3			
		0.6			
				0.5 x 10 ⁸	0.02 0.76 0.71
	Ceepryn	0.3			
		0.6			
				0	-
				0	-
<u>P. fluorescens</u>	Perchloric acid	0			
		0.3			
		0.6			
				4.6 x 10 ⁸	0.20 0.73 0.71
	Ceepryn	0.3			
		0.6			
				0	-
				0	-
Medium control	Perchloric acid	0			
		0.3			
		0.6			
				0	0.81 0.79 0.75
	Ceepryn	0.3			
		0.6			
				0	-
				0	-



of its interaction with ammonium molybdate to form a precipitate.

Perchloric acid is a rather promising poison. It is a strong germicide and does not interact with ingredients in RM9 to form precipitates. Growth tests have shown that a slightly higher concentration of perchloric acid is necessary to inhibit the growth of yeast, and Saccharomyces cerevisiae. (Table No. 9). Results of E. coli cultures treated with various poisons are shown in Table No. 10. Phenol at 0.01% in RM9 medium was ineffective in inhibiting the E. coli growth and was not further investigated.

3. Theoretical Consideration of Phosphate Uptake

The phosphate content of average bacteria is 2% and the dry weight of 10^7 cells is $6.7 \mu\text{g}$. (9). Thus the dry weight of phosphorus in the 10^7 cells will be $0.134 \mu\text{g}$. The RM9 contains 5 mg. K_2HPO_4 per liter or approximately 1 mg. $\text{PO}_4\text{-P}$ per liter. This value is equivalent to 1000 ~~pg~~ μg . per liter or $1 \mu\text{g}$. per ml. Since 10^7 cells required $0.134 \mu\text{g}$. of $\text{PO}_4\text{-P}$ to grow, 1 mg. $\text{PO}_4\text{-P}$ per liter is sufficient to support the growth of more than 10^7 cells per ml. of medium.

4. Pure Culture Studies

a. M9 Medium

(1) Effect of Aeration On Phosphate Uptake

In order to test the possibility of increasing phosphate uptake, the microorganism, 'Serratia marcescens' in 40 ml. test medium with various concentrations of phosphate, was aerated during incubation at 37°C . Plate counts showed that: there were 4.8×10^9 cells/ml. As

Table No. 9 - Inhibitory effect of Bard-Parker and 70% perchloric acid on Saccharomyces cerevisiae.

<u>SAMPLE</u>	<u>CELL DENSITY (NO./ML)</u>	
	<u>0 TIME</u>	<u>24 HOURS</u>
S. <u>cerevisiae</u> in 40 ml. RM9	3.75 x 10 ⁴	5.0 x 10 ⁴
S. <u>cerevisiae</u> in RM9 + 0.3 ml. BP	3.75 x 10 ⁴	0
S. <u>cerevisiae</u> in RM9 + 0.6 ml. BP	3.75 x 10 ⁴	100
S. <u>cerevisiae</u> in RM9 + 0.3 ml. perchloric acid*	3.75 x 10 ⁴	1.5 x 10 ³
S. <u>cerevisiae</u> in RM9 + 0.6 ml. perchloric acid*	3.75 x 10 ⁴	0
S. <u>cerevisiae</u> in RM9 + 1.0 ml. perchloric acid''	3.75 x 10 ⁴	0

* 0.3 ml. 70% perchloric acid = 0.525%
 0.6 ml. 70% perchloric acid = 1.05%
 1.0 ml. 70% perchloric acid = 1.75%

Table No. 10 - Effect of poisons on microbial growth, phosphate analysis, and ATP production.

SAMPLE TREATMENT	CELL DENSITY		PO ₄ -P		ATP NET RESPONSE (NA)*	
	0 HR.	24 HR.	0 HR.	24 HR.	0 HR.	24 HR.
Medium Blank		0				
<u>E. coli</u> Culture	4.6 x 10 ⁶ /ml	5.5 x 10 ⁸ /ml	0.81 mg P/L	0.84 mg P/L	0.02 na	0.02 na/2 ml
Perchloric Acid 0.175%	4.6 x 10 ⁶ /ml	0	0.94	0.92	0.3	0.13
0.06%	4.6 x 10 ⁶ /ml	0	0.92	0.88	0.7	0.07
0.03%	4.6 x 10 ⁶ /ml	1.4 x 10 ⁴ /ml**	0.80	0.66	0.3	0.78
Toluene 0.6 ml.	4.6 x 10 ⁶ /ml	0	0.92	0.88	0.09	0.12
0.3 ml.	4.6 x 10 ⁶ /ml	0	0.88	0.86	0.08	0.11
0.1 ml.	4.6 x 10 ⁶ /ml	2 x 10 ⁷ **	0.89	0.80	0.6	10.75
Bard-Parker 0.6 ml.	4.6 x 10 ⁶ /ml	0	0.97	1.08	0.5	0.15
0.3 ml.	4.6 x 10 ⁶ /ml	0	0.92	0.85	0.6	0.18
0.1 ml.	4.6 x 10 ⁶ /ml		0.90	0.76	0.08	0.27
Phenol 0.01%	4.6 x 10 ⁶ /ml	2 x 10 ⁷	0.92	0.04	0.5	17.0
0.03%	4.6 x 10 ⁶ /ml	2 x 10 ⁷	0.90	0.04	1.7	5.0
0.06%	4.6 x 10 ⁶ /ml	1.7 x 10 ⁷	0.81	0.04	1.8	9.75

* 2 ml. culture was filtered on a membrane filter which was then extracted with 1 ml. 90% DMSO.
 ** approximate

shown in Figure No. 9, the results are quite similar to those obtained with the other organisms (see Figures No. 7 and No. 8). The phosphate at 1 mg. per liter **was** exhausted in 24 hours.

When phosphate uptake was determined for Aerobacter aerogenes, a smaller number of cells (2.3×10^6 cells/ml.) were used. The flasks were aerated by passing in air through gas diffusion tubes. The results obtained are summarized in Table No. 11. The zero time reading for the higher phosphate concentration seems dubious, but one can still see the usual pattern: slight or no phosphate uptake for the first few hours and then appreciable uptake by the end of **24** hours, with essentially all of the phosphate removed from low phosphate medium.

(2) Effect of Small Inoculum and Aeration On Phosphate Uptake

To increase sensitivity even further, phosphate uptake **was** measured when even fewer **cells** were present. S. marcescens was treated at a level of 2.7×10^6 cells/ml. and A. aerogenes was tested at a level of 4×10^5 cells/ml. The results are summarized in Table No. 12. The pattern is fairly consistent with previous results, particularly with regard to the return of phosphate to the medium in the 24- to 48-hour period, but there is more phosphate uptake than previously observed. **It** does not seem reasonable to attribute this to the decreased number of cells used in this experiment. **It** seems more likely that this resulted from an increased aeration rate. Another interesting observation is that the uptake was essentially independent of initial phosphate concentration in the medium.

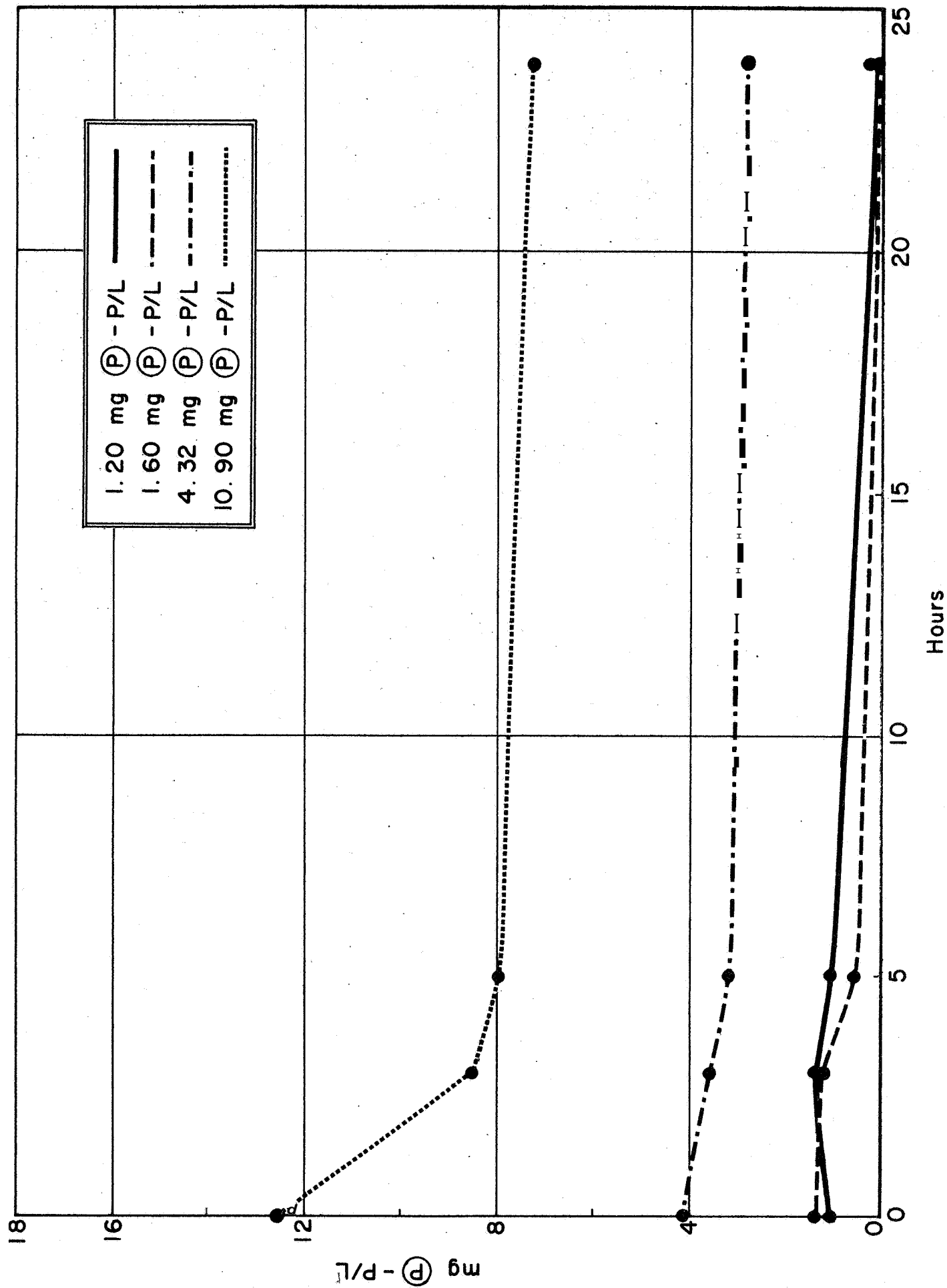


Figure No. 9 - Phosphate uptake by S. marescens, various concentrations of phosphate in medium.

Table No. 11 - Phosphate uptake by A. aerogenes in M9 medium with 0.2% glucose and two concentrations of phosphate.

SYSTEM	PO ₄ -P MG. PER LITER			
	<u>0</u> hr.	<u>3</u> hr.	<u>5</u> hr.	<u>24</u> hr.
2.0 mg. PO ₄ -P/L	1.32	1.27	0.75	0.07
5.0 mg. PO ₄ -P/L	3.67	4.22	4.17	1.40

Table No. 12 - Phosphate uptake by A. aerogenes (4×10^5 cells/ml)
and S. marcescens (2.7×10^5 cells/ml).

SYSTEM	PO ₄ -P MG. PER LITER				
	<u>0</u>	<u>3</u>	<u>6</u>	<u>24</u>	<u>48</u>
	hr.	hr.	hr.	hr.	hr.
<u>A. aerogenes</u> — 4.75 mg. PO ₄ -P/L	4.30	4.15	4.00	1.65	2.87
<u>A. aerogenes</u> 14.30 mg. PO ₄ -P/L	13.90	13.30	12.65	11.70	13.60
<u>S. marcescens</u> 4.75 mg. PO ₄ -P/L	4.87	4.30	4.15	2.72	2.80
<u>S. marcescens</u> 14.30 mg. PO ₄ -P/L	14.30	13.50	13.00	11.75	13.40

Using the increased aeration rate, low numbers of E. coli (5.0×10^4 cells/ml.) and A. aerogenes (4.9×10^6 cells/ml.) were tested for phosphate uptake. The results are given in Table No. 13. These lower numbers of cells give good phosphate uptake within 24 hours. At either 1.30 mg. of $\text{PO}_4\text{-P}$ per liter or 0.57 mg. of $\text{PO}_4\text{-P}$ per liter there is essentially total uptake.

In the next experiment, even lower numbers of cells (ca. 1.3×10^2 cells/ml) were tested for their ability to take up phosphate from a low phosphate medium. As can be seen from the results in Table No. 14, even these few cells assimilated detectable amounts of phosphate. The early uptake (at five hours) seems to contradict some of the previous results. It is believed that this may have resulted from the fact that those cells used were still in the logarithmic phase of growth, whereas the previous cells were in the lag phase at zero time.

(3) Addition of Various Substrates to Medium on Rate of Phosphate Uptake

A culture of E. coli grown at 24 hours was treated in a fashion similar to that described in the last section except that glucose was substituted by formate, lactate, or glycine (all at 0.2%). The results are shown in Figure No. 10. The initial uptake of phosphate was almost as good in the absence of substrate as with the addition of substrate. Presumably, this indicates that there was sufficient substrate remaining in the cells to allow metabolism to proceed for a few hours. However, the uptake in a 24-hour period was enhanced when either D-glucose or lactate was added.

Table No. 13 - Phosphate uptake by E. coli (5.0 x 10⁴ cells/ml)
and A. aerogenes (4.9 x 10⁶ cells/ml).

SYSTEM	PO ₄ -P MG. PER LITER				
	HOURS				
	<u>0</u>	<u>3</u>	<u>5</u>	<u>24</u>	<u>48</u>
<u>E. coli</u> 0.57 mg. PO ₄ -P/L	0.45	0.25	0.58	0.02	0.00
<u>E. coli</u> 1.30 mg. PO ₄ -P/L	1.15	0.99	1.13	0.00	0.02
<u>A. aerogenes</u> 0.57 mg. PO ₄ -P/L	0.54	0.16	0.16	0.00	0.00
<u>A. aerogenes</u> 1.30 mg. PO ₄ -P/L	1.17	1.00	0.95	0.00	0.02

Table No. 14 - Phosphate uptake by E. coli, S. marcescens
and A. aerogenes from low phosphate medium:

SYSTEM	PO ₄ -P MG. PER LITER			
	HOURS			
	0	3	5	24
Control	0.70	-	-	0.70
<u>E. coli</u> 1.17 x 10 ² cells/ml	0.72	0.75	0.37	0.20
<u>S. marcescens</u> 1.30 x 10 ² cells/ml	0.66	0.59	0.17	0.10
<u>A. aerogenes</u> 1.42 x 10 ² cells/ml	0.64	0.61	0.47	0.42

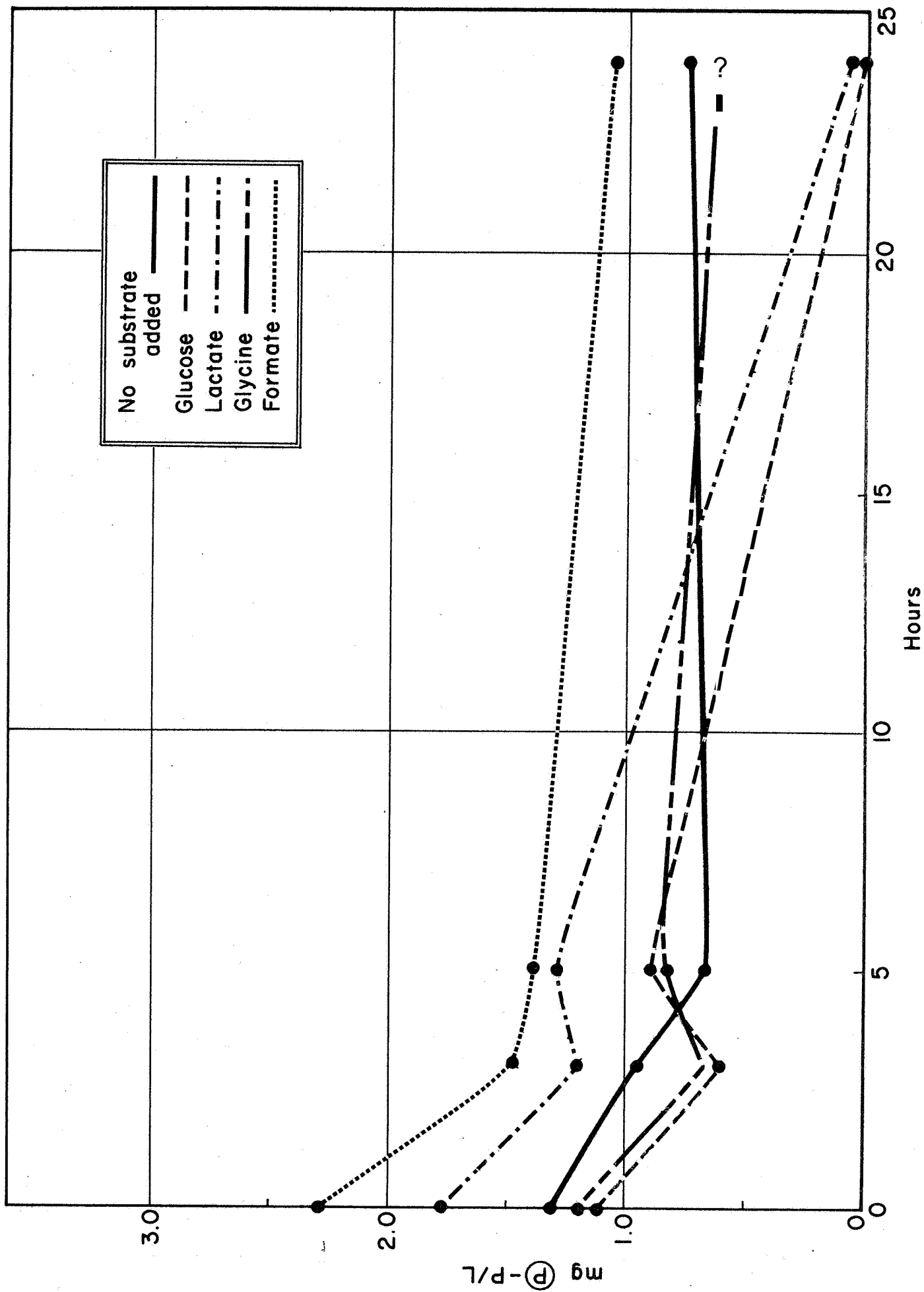


Figure No. 10 - Effect of substrates on phosphate uptake by *E. coli*

Two other parameters were checked in the course of this experiment: (1) pH of filtrates and (2) plate counts after 24 hours of growth in the various supplemented media. The pH results are given in Table No. 15. They agree with the phosphate uptake results in showing that the glucose and lactate-supplemented systems had the greatest amount of metabolic activity. The plate counts were not quite consistent with this, however. At the start of the experiment each system had 6.1×10^4 cells/ml. After 24 hours, the lactate system had more cells (5.2×10^8 cells/ml.) than any other system and the other supplemented media cultures contained approximately equal cell populations (glucose = 7.0×10^7 ; glycine = 5.0×10^7 ; formate = 2.5×10^7 cells/ml.). Of course, metabolic activity and reproduction of cells are far from equivalent.

This type of experiment was repeated using a lesser number of cells at the start (2×10^3 E. coli per ml.). As seen in Figure No. 11, the glucose-supplemented medium shows good phosphate uptake, even at five hours.

(4) Age of Inoculum and Phosphate Uptake

An E. coli cell suspension was inoculated into 40 ml. of low-phosphate (1 mg. PO_4 -P per liter) M9 medium containing 0.2% glucose. After 21 hours of incubation, a portion of the culture was transferred to fresh, low-phosphate medium to initiate a second culture. Three hours later, the first culture became 24 hours old (stationary phase) and its subculture became three hours old (lag phase). The cell density in each culture was roughly determined from an O.D. curve. One ml. (approximately 3.5×10^8 cells) of the 24-hour culture and 1.0 ml. (approximately 1×10^8 cell) of the three-hour culture were

Table No. 15 - The pH of filtrates of E. coli grown in M9 medium with **1 mg.** of $\text{PO}_4\text{-P/L}$ and various supplements*.

SYSTEM	HOURS			
	<u>0</u>	<u>3</u>	<u>5</u>	<u>24</u>
M9 (medium only)	6.9	-	-	6.9
M9 with <u>E. coli</u>	7.0	6.9	6.5	6.9
M9 + glucose (medium only)	7.0	-	-	7.4
M9 + glucose with <u>E. coli</u>	7.3	6.9	6.9	5.0
M9 + formate (medium only)	7.4	-	-	7.4
M9 + formate with <u>E. coli</u>	7.1	7.2	6.6	7.0
M9 + lactate (medium only)	7.2	-	-	7.9
M9 + lactate with <u>E. coli</u>	7.5	7.4	7.1	6.0
M9 + glycine (medium only)	7.2	-	-	7.2
M9 + glycine with <u>E. coli</u>	7.2	7.1	6.7	7.3

*

All supplements are at 0.2%.

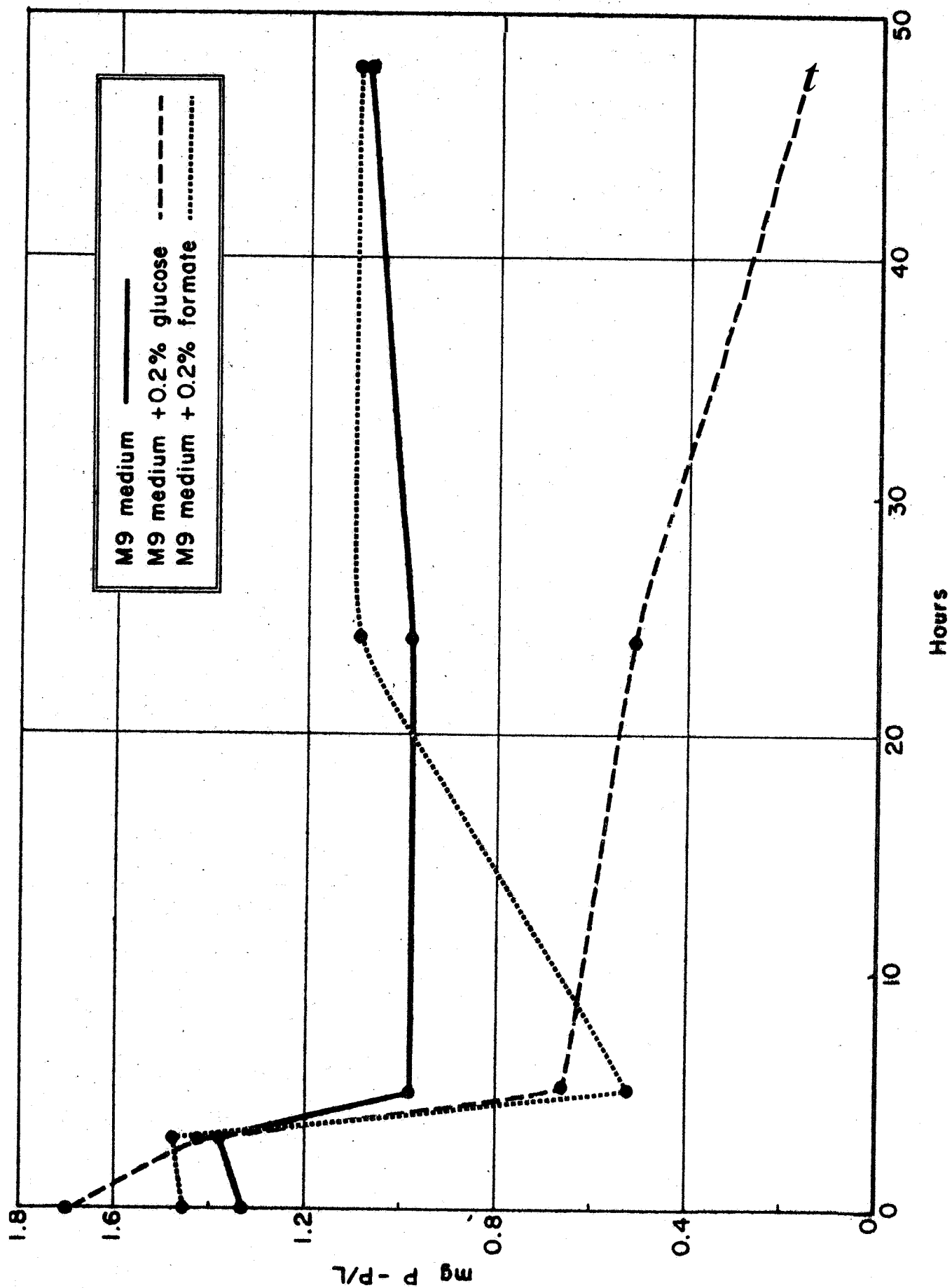


Figure No. 11 - Phosphate uptake by *E. coli* in M9 medium (1.3 mg (P) - P/L) and in medium supplemented with 0.2% glucose or formate.



centrifuged. The 24-hour cells were washed three times with sterile saline solution, resuspended in saline and serially diluted to prepare 0.1 ml. aliquots containing approximately 1.6×10^6 , 1.6×10^5 , and 1.6×10^4 cells. These aliquots were inoculated into 40 ml. portions of low-phosphate M9 medium to give 4×10^4 , 4×10^3 , and 4×10^2 cells per ml., respectively. One-tenth ml. aliquots of the three-hour inoculum containing 1×10^6 , 1×10^5 , and 1×10^4 were similarly inoculated into 40 ml. low-phosphate M9 medium to yield 2×10^4 , 2×10^3 , and 2×10^2 cells per ml., respectively. All cultures were prepared in duplicate. Duplicate controls containing 4×10^4 cells per ml., in the case of the 24-hour culture, and 2×10^4 cells, in the case of the three-hour culture, were treated with 2,4-dinitrophenol (DNP) at 0.66 mM. All the cultures were aerated at a rate of 92 ml. per minute while incubating at 37°C . Actual cell counts were determined by Tryptic Soy Agar (TSA) plates made from aliquots of the inocula.

Aliquots from each incubating culture were taken at 0 time, five hours, and 24 hours. They were filtered by membrane filter and the filtrate was assayed for dissolved orthophosphate. TSA counts were made at 0 time, and at 24 hours. The results are shown in Figure No. 12. In all cases, except the controls, phosphate uptake was demonstrated in five hours, the earliest sampling after time 0.

Phosphate uptake was always higher in the physiologically younger cultures. In both the three-hour and 24-hour inocula cultures, the greatest phosphate uptake consistently occurred in cultures of smallest inoculum. Since both types of cultures attained cell numbers

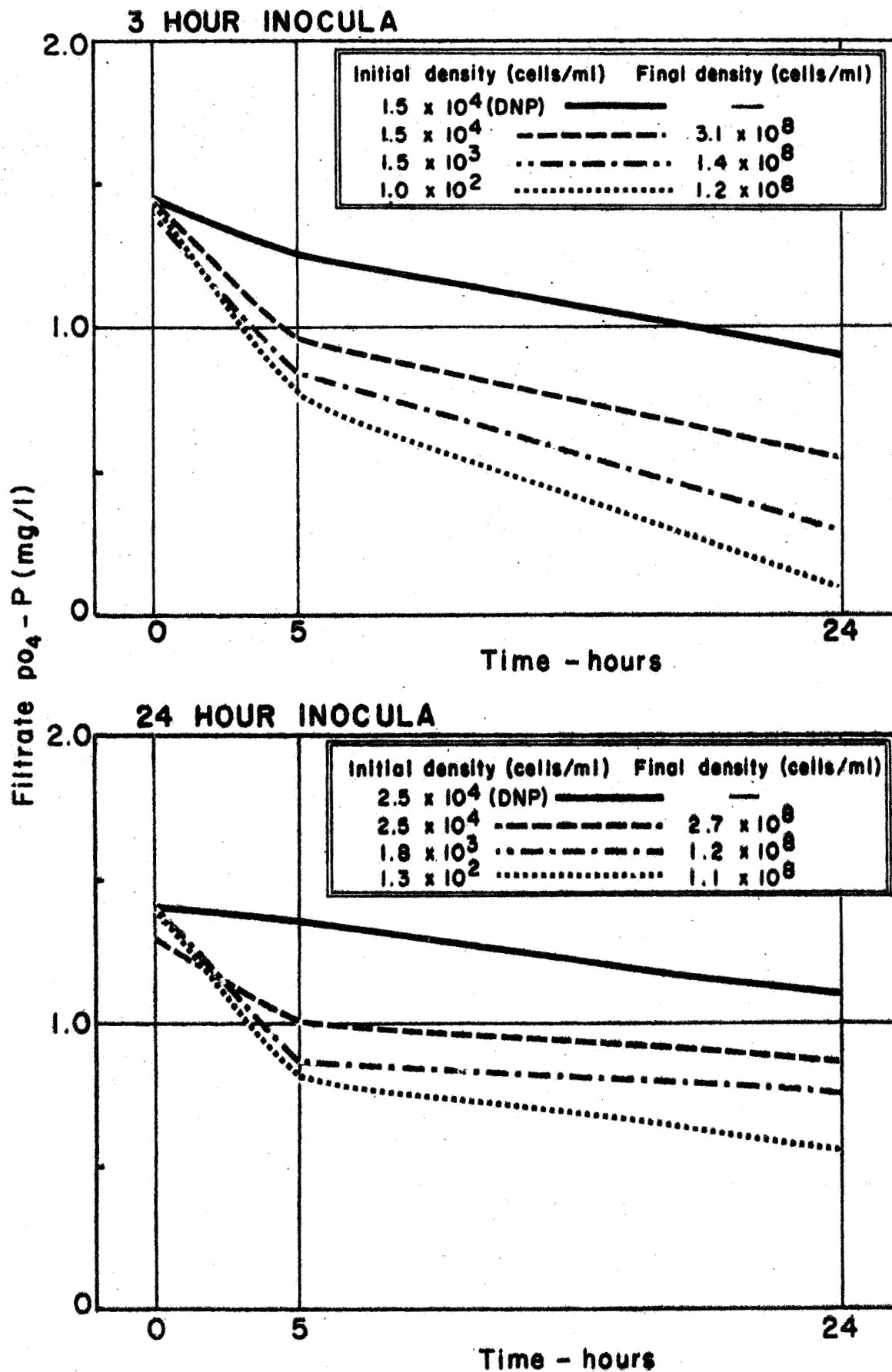


Figure No.12 - Orthophosphate uptake by aerating (92 ml/min) cultures of E. Coli seeded from 3 hour and 24 hour cultures grown in low-phosphate M9.

of 10^8 per ml., more cell mass had been produced from the lower inocula which, hence, required more phosphate. The lesser phosphate uptake shown by the DNP-treated cultures **was** the result of uncoupling of oxidative phosphorylation, demonstrating that the uptake was biological in nature. The small amounts of phosphate taken up by these cultures can probably be ascribed to substrate phosphorylation by the inhibited cells.

To compare phosphate uptake between the aerated and stationary cultures under the influence of age of the inoculum, *E. coli* was studied again under an experimental design similar to that described above. However, this time all the cultures were incubated in a static condition at 37° C. Bard-Parker (BP) was used in the controls (3.0 ml. BP + 37.0 ml. M9 medium). Under these conditions, clearly demonstrable phosphate uptake occurred between the five- and 24-hour samplings. Details are given in Figure No. 13.

The age of the inoculum did not influence phosphate uptake as it did in the case of the aerated cultures. Contrary to the observations on aerated cultures, phosphate uptake varied directly with size of inoculum.

The inoculated controls poisoned with BP showed no uptake of phosphate. Instead, their dissolved phosphate levels were consistently higher than those of the sterile controls, demonstrating that phosphate leaked out from the poisoned cells almost immediately. This, even more forcefully than the DNP, showed the biological nature of the uptake.

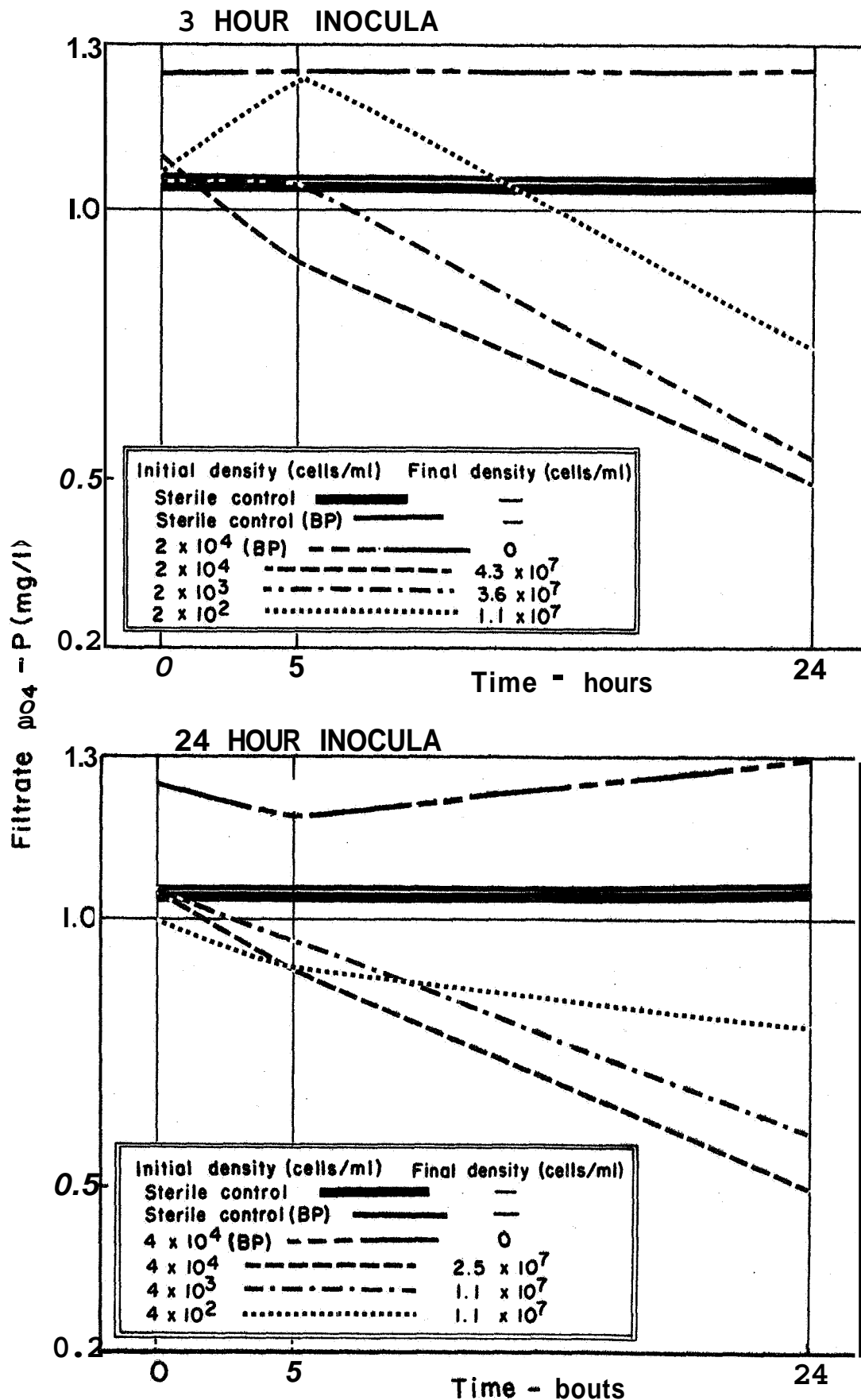


Figure No.13 – Orthophosphate uptake by non-aerating cultures of E. Coli seeded from 3 hour and 24 hour culture. grown in low-phosphate M9.

(5) Preadaptation of Cell Inoculum and Phosphate Uptake

To prepare inocula for the test, a stock culture of P. fluorescens was transferred to M9 medium and also to Tryptic Soy Broth (TSB). After 48 hours, inocula from each media were prepared and adjusted to 7×10^7 cells per ml. for TSB inoculum and 8.2×10^7 cells per ml. for M9 inoculum. One ml. each was added to 40 ml. of medium. Glucose concentrations were 0.002, 0.02 and 0.2% for each inoculum culture. As a control, 1.0 ml. of Bard-Parker germicide was added to 40 ml. of each medium. The results are shown in Figure No. 14. The inocula preadapted in different media did not affect the pattern of phosphate uptake. In fact, there was very little difference between them. Glucose at 0.02% and 0.2% appeared to be beneficial for phosphate uptake but for 0.002% glucose, there was very little phosphate uptake despite the fact that there was evidence of growth in the culture.

(6) Phosphate Uptake by Anaerobes

Attempts to grow anaerobes, Clostridium sporogenes and Ct. tetanmorphum, in either M9 or M11 with reduced phosphate concentrations, were not successful. Anaerobic conditions were produced by several standard techniques, several levels of phosphate were tested, etc. However, when thioglycollate was added to the M11 it was possible not only to demonstrate growth of the organism, but it was also possible to demonstrate phosphate uptake (see Figure No. 15). Plate counts have shown that C1. sporogenes was inoculated at 2.8×10^6 cell/ml. and increased to 1.8×10^8 cells/ml. after 24 hours; the corresponding values for C1. tetanmorphum were 4.8×10^6 and 2.8×10^8 cells/ml., respectively.

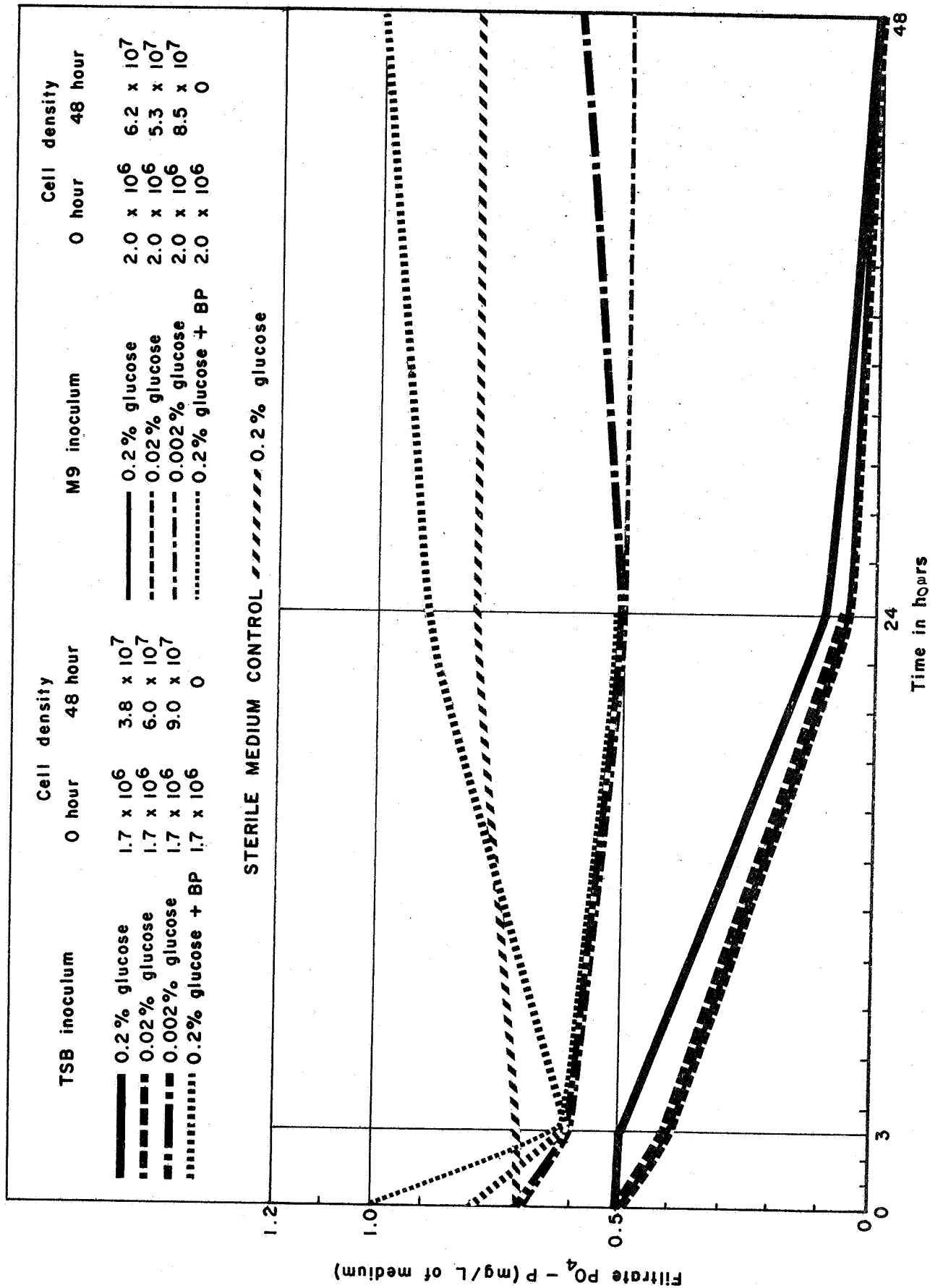


Figure 14 — Phosphate uptake by *Pseudomonas fluorescens* in M9 media pNs different concentrations of glucose under aerated (20 ml/min/40 ml) and 26° c. conditia, the inocula being pre-adapted in synthetic M9 and in complex Tryptic Soy Broth media.

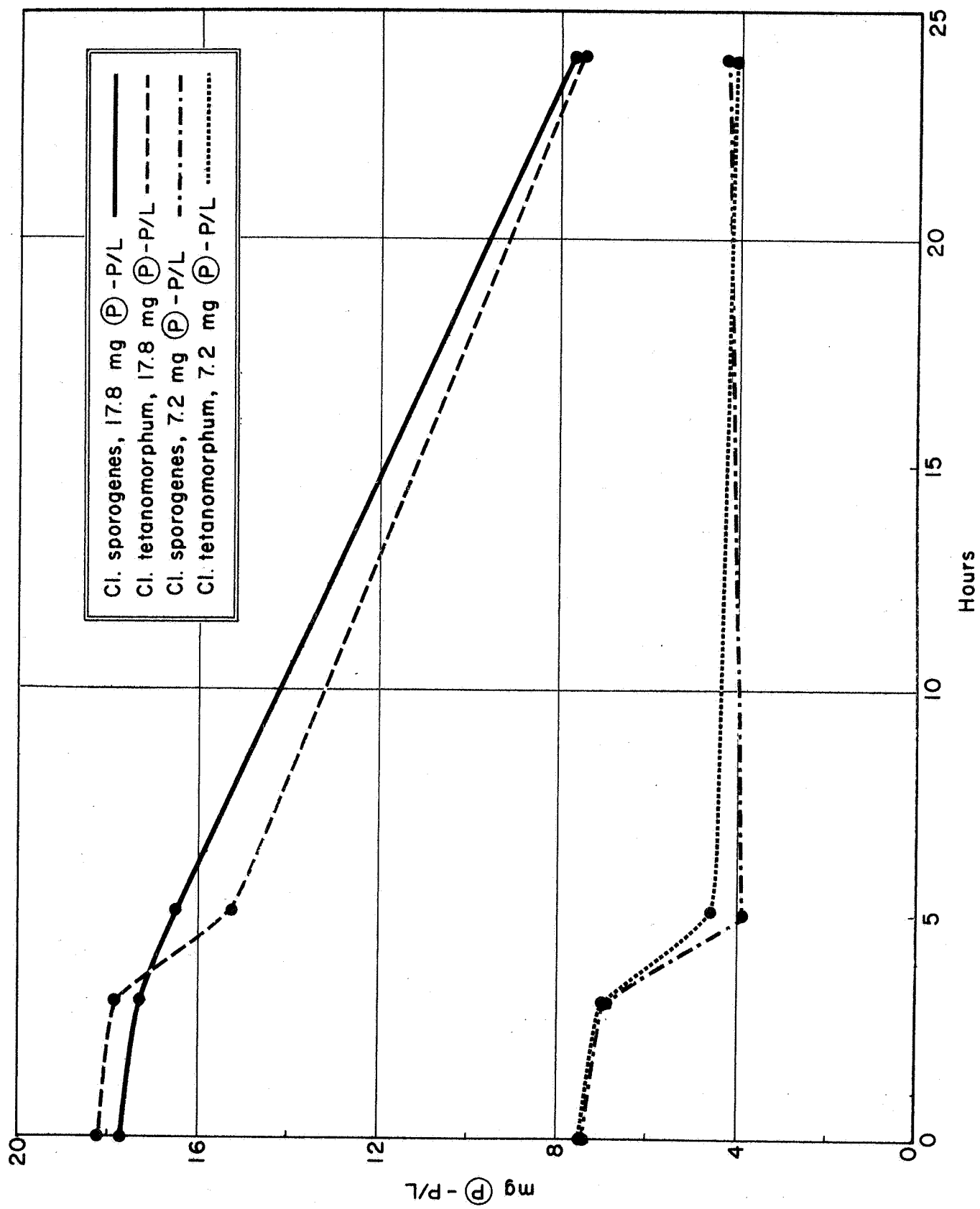


Figure No.15 — Phosphate uptake by anaerobes in MII supplemented with 0.2% glucose and 0.1 g thyoglycollate/L; various concentrations of phosphate.



A further test was made on C1. tetanmorphum by growing it in M9 plus thioglycollate. C1. tetanmorphum was subcultured from cooked meat medium into low-phosphate (1.0 mg. $\text{PO}_4\text{-P}$ per liter) M9 containing 0.2% glucose and 0.01% Na-thioglycollate (M9-TG) under anaerobic conditions obtained by flushing with N_2 gas 10 minutes before and after inoculation. The gas lines were closed afterward to prevent diffusion of oxygen into the culture medium. A 24-hour culture containing approximately 5×10^7 cells per ml. was washed and serially diluted with M9-TG. One-tenth ml. aliquot of the diluted culture was added to 40 ml. M9-TG medium to obtain approximate cell concentrations of 1×10^4 , 1×10^3 , and 1×10^2 per ml., and anaerobiosis was reestablished. Since this anaerobic organism is slower growing than E. coli, the 24-hour culture was used as the "young inoculum." The parent culture served as the "old inoculum" after 48 hours. The approximate density of the 48-hour inoculum was 9×10^8 cells per ml. Zero time cell numbers in 40 ml. M9-TG were 1×10^4 , 1×10^3 , and 1×10^2 per ml. for both inocula. The results are shown in Figure No. 16.

Phosphate uptake was noticed at five-hour incubation from cultures of 24-hours inoculum. The corresponding cultures from 48-hours inoculum did not show any phosphate uptake. No final cell counts were made and it is possible that the cells died. The fact that phosphate leakage varied directly with size of the inoculum indicates this was the case. The pattern of phosphate uptake in the 24-hour inocula cultures followed the aerated E. coli culture, the greatest uptake being exerted by the culture derived from the smallest inoculum.

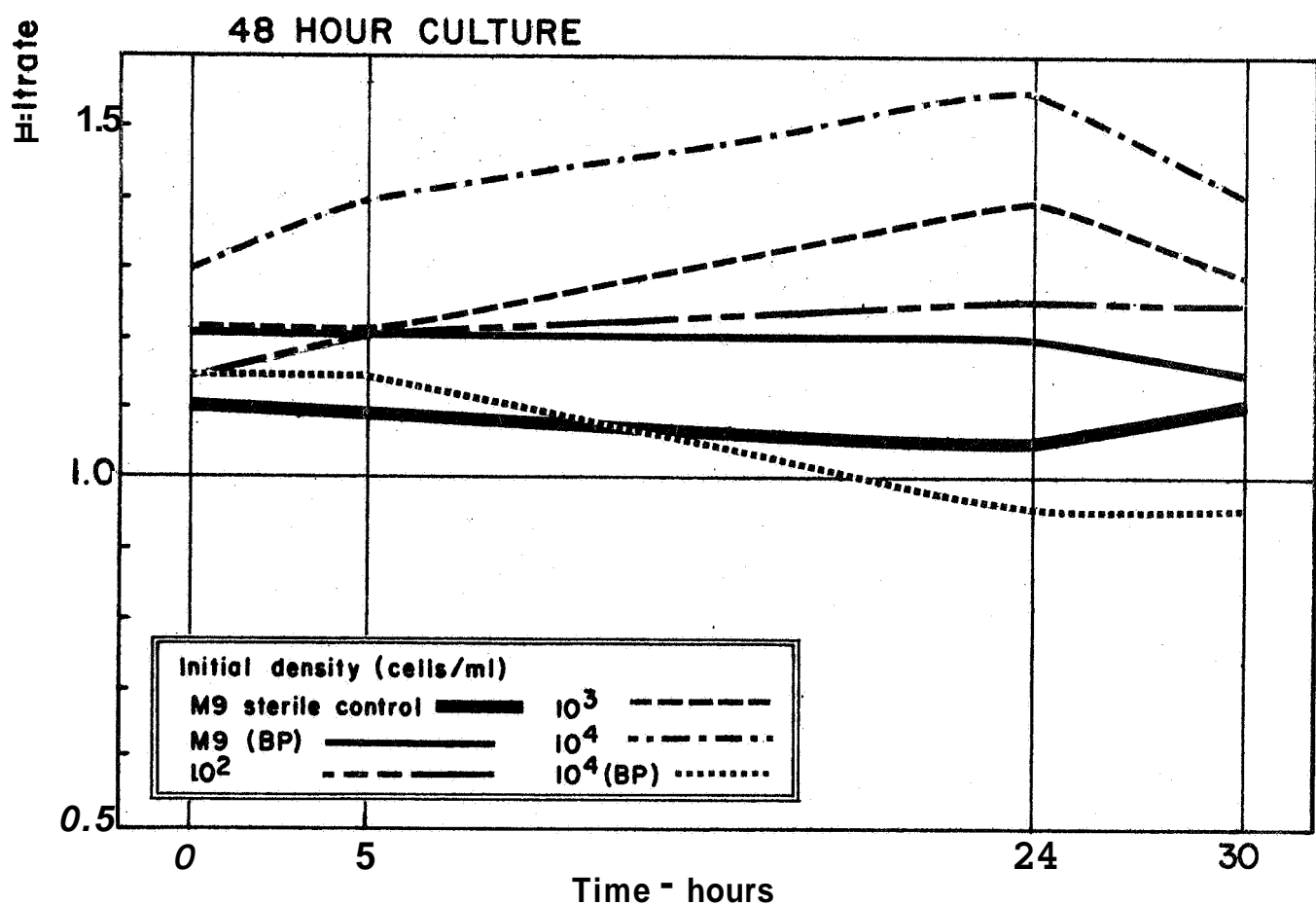
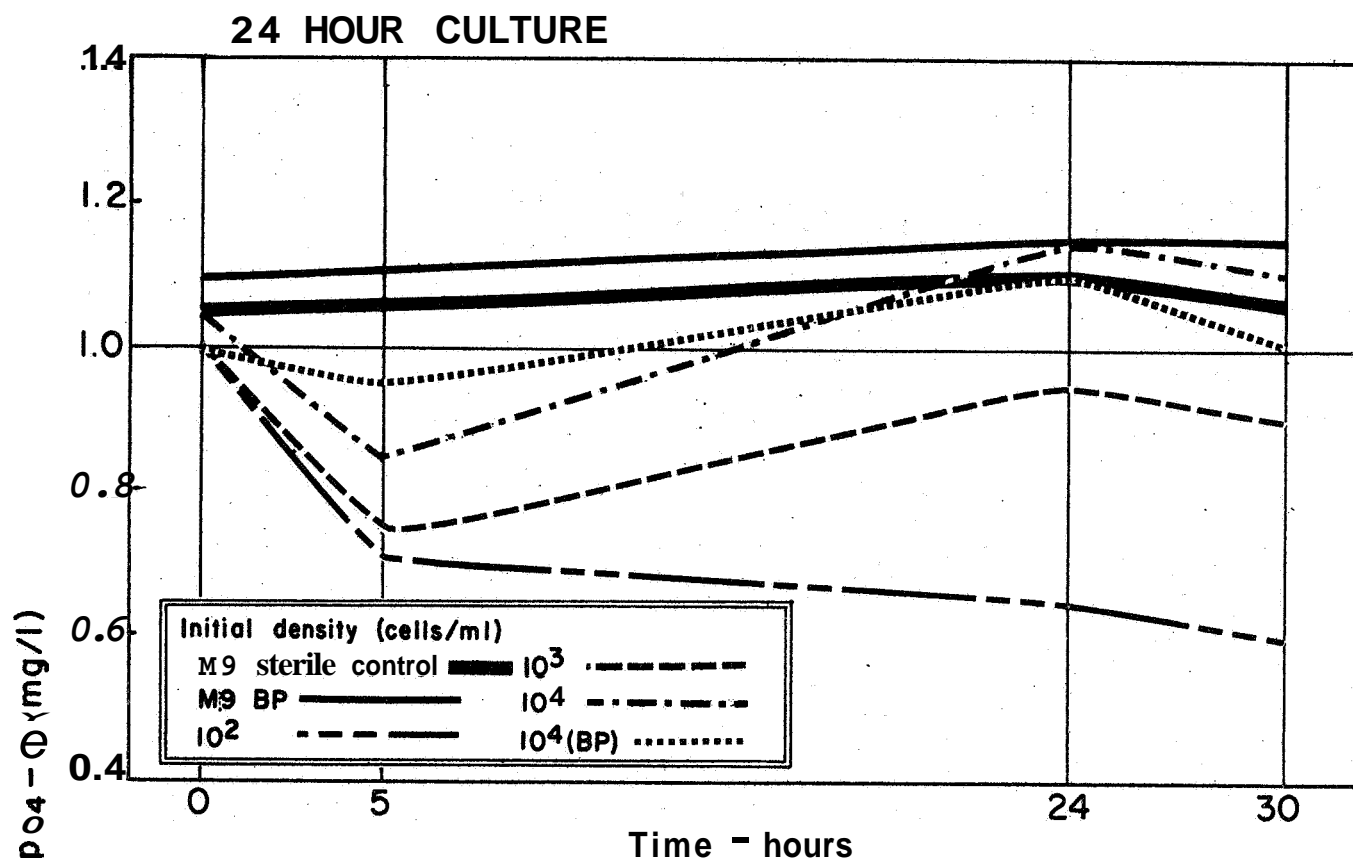


Figure No.16 - Orthophosphate uptake by cultures of Cl. tetanomorphum seeded from 24 hour and 48 hour cultures grown in M9-TG.



The mode of action of Bard-Parker poison on anaerobic

C1. tetanomorphum seems to be different from that operating on the facultative E. coli. No clear pattern of phosphate leakage was observed in the former species.

b. RM9 Medium

(1) Phosphate Uptake by Conventional Methods

The formula for RM9 medium is shown in Table No. 4. This is a revised M9 medium which contains ingredients for both aerobic as well as anaerobic growth.

Both aerobic P. fluorescens and E. coli inocula were grown on Tryptic Soy Broth overnight at 30° C. One-tenth ml. of washed inoculum of each microorganism was added to 40 ml. of medium to give 1.3×10^4 and 3.7×10^4 cells per ml. of medium for the respective cultures. Experimental conditions and results are shown in Table No. 16. The medium did support growth, and phosphate uptake was evident for both cultures. The aerobic P. fluorescens took up a considerable amount of phosphate even without aeration. E. coli exhausted the phosphate in 24 hours. It was noted, however, that the phosphate was higher in five hours than in zero-hour cultures for both organisms, including the medium control. The reason was attributed to possible contamination of exogenous organic phosphate compounds from soil extract which, when decomposed and released during the incubation or storage, increased the phosphate content in the media. The five-hour filtrate was generally stored at 5° C. overnight in the presence of chloroform because the analysis was generally due at the end of a working day. This may have provided conditions for

Table No. 16 - Phosphate uptake by *Pseudomonas fluorescens* and *Escherichia coli* at room temperature (26° C.), static condition in revised M9 media*.

ORGANISMS	INITIAL CELL DENSITY/ML (IN 40 ML.)	TREATMENTS (IN 40 ML.)	POL-P IN MG/L			PLATE COUNT AT 24 HOURS ml.
			0 HOUR	5 HOUR	24 HOUR	
<u>P. fluorescens</u>	1.3 x 10 ⁴	1. Experimental	1.12	1.28	0.67	4.5 x 10 ⁷
		2. + 0.1 ml. Bard-Parker	1.14	1.30	1.32	0
		3. + 0.1% HgCl ₂	1.16	1.44	1.50	0
<u>E. coli</u>	3.7 x 10 ⁴	1. Experimental	1.12	1.30	0.00	3.7 x 10 ⁸
		2. + 0.1 ml. Bard-Parker	1.26	1.34	1.36	0
		3. + 0.1% HgCl ₂	1.26	1.40	1.44	0
--	--	Medium Control	1.14	1.40	1.32	0

*RM9: per liter: K₂HPO₄, 5.0 mg.; NH₄NO₃, 0.2 g.; MgSO₄·7H₂O, 0.08 g.; NaCl, 0.1 g.; soil extract, 100 ml. (per 900 ml.); glucose, 0.2 g.; Na-thioglycollate, 0.002 g.; Tris, 6.0 g., pH 7.0



releasing phosphate from the organic compounds. The 24-hour filtrate from medium control, although not refrigerated, still contained phosphate higher than in the zero-hour sample. This probably was due to prolonged incubation. To prove that incubation time may affect the release of phosphate from a control medium, three sets of duplicate samples were prepared and incubated at 25° C., 37° C., and 50° C., respectively. Phosphate analysis was made at 0, 5, and 24 hours. The results are shown in Table No. 17. The highest phosphate was in the five-hour samples and lower in the 24-hour samples.

(2) Phosphate Uptake by Conventional and Radioisotope Analyses

All the phosphate analyses reported thus far in the uptake study were done solely by the conventional ammonium molybdate stannous chloride method. An attempt to use ¹⁴C-triethylamine method is now reported. This method could permit a single photomultiplier tube to be employed to detect microbial activities in phosphate uptake, sulfate uptake, and ATP production.

E. coli and P. fluorescens previously grown on Tryptic Soy Broth were washed, resuspended in saline, and inoculated into 40 ml. RM9 medium to yield cell densities of 1.5×10^4 and 5.0×10^3 per ml., respectively. Additional E. coli cultures, plus HgCl₂ poison and a medium control were included for studies on phosphate uptake. Table No. 18 shows the results of this experiment. The initial and final phosphate concentrations in mg. per liter for E. coli, P. fluorescens, poisoned E. coli, and medium control were, respectively, 0.93 and 0.02; 0.89 and 0.66; 1.25 and 1.11; and 0.92 and 1.00. Uptake is

Table No. 17 - Effect of time and temperature on orthophosphate analysis in RM9 control medium.

<u>TEMPER- ATURE</u> ° C.	<u>SAMPLE</u>	<u>0 HOUR</u>		<u>5 HOUR</u>		<u>24 HOUR</u>	
		<u>PO₄-P MG/L</u>	<u>AVER - AGED</u>	<u>PO₄-P MG/L</u>	<u>AVER - AGED</u>	<u>PO₄-P MG/L</u>	<u>AVER - AGED</u>
25	1	0.90	0.88	1.03	1.03	1.00	0.97
	2	0.86		1.03		0.95	
37	1	0.88	0.88	1.03	1.03	0.96	0.99
	2	0.88		1.03		1.02	
50	1	0.86	0.87	1.05	1.05	1.03	1.02
	2	0.88		1.05		1.02	

Table . 1² - Analyses of ATP and phosphate by ammonium molybdate and triethylamine-¹⁴C methods in Escherichia coli and Pseudomonas fluorescens at room temperature (26° C.), static condition in revised M9 medium.

TREATMENT	ANALYSES	INITIAL CELL DENSITY per/ml.	TIME			FINAL CELL DENSITY per ml.
			0 HOUR	5 HOUR	24 HOUR	
<u>E. coli</u>	ATP (na.)	1.5 x 10 ⁴	0	0.04	8.10	4 x 10 ⁸
	PO ₄ -CM (mg/l)*		0.93	0.90	0.02	
	PO ₄ -TEA (cpm)**		166	133	43	
<u>P. fluorescens</u>	ATP (na.)	5.0 x 10 ³	0	0.03	3.24	1.3 x 10 ⁷
	PO ₄ -CM (mg/l)		0.89	0.92	0.66	
	PO ₄ -TEA (cpm)		134	122	96	
<u>G. coli</u> + 0.1 ml. HgCl ₂	ATP (na.)	1.5 x 10 ⁴	0	0.01	0.05	0
	PO ₄ -CM (mg/l)		1.25	1.18	1.11	
	PO ₄ -TEA (cpm)		119	154	135	
Medium alone	ATP (na.)	-	0	0.04	0.04	0
	PO ₄ -CM (mg/l)		0.92	0.94	1.00	
	PO ₄ -TEA (cpm)		148	142	144	

* PO₄-CM is phosphate assayed by conventional ammonium molybdate-stannous chloride method.

** PO₄-TEA is phosphate assayed by ¹⁴C-triethylamine method.



shown only in non-poisoned cultures. The ^{14}C -triethylamine method, expressed in **cpm**, was parallel with the conventional method.

5. Mixed Culture Studies

The P. fluorescens and C. tetanomorphum were grown separately on Tryptic Soy Broth and Fluid Thioglycollate Media, respectively. They were washed and resuspended in saline. Individual pure cultures and a mix of both cultures of 1:1 (by volume) were inoculated into 40 ml. of RM9 media. Initial cell densities and the results of phosphate uptake are shown in Table No. 19.

Both pure and mixed cultures were grown at 26°C . under static conditions. The mixed culture had the highest uptake and the Pseudomonas culture was next. No uptake was observed in the pure Clostridium culture. The viable cell number of this culture, in fact, dropped from an initial 1×10^5 per ml. to a final 2.3×10^4 per ml. This indicates that some of the cells might have died during the incubation. The Clostridium number in the mixed culture, however, increased twofold within 24 hours.

6. Soil Sample Studies

a. Interference Problems

The objection of using soil suspension as direct inoculum to add to a testing medium for ATP production and for phosphate and sulfate uptake is that some soils are capable of taking up phosphate and sulfate by their anion exchange capacity and thus may cause a false positive result. Furthermore, the soil particles retained along with the microbial cells by a membrane filter may adsorb the radiation given off from radioactive sulfate in the cells. This would reduce the sensitivity for detecting sulfate uptake. To show whether the uptake

Table No. 19 - Phosphate uptake by pure and mixed cultures of Pseudomonas fluorescens and Clostridium tetanomorphum at room temperature (26° C.), static condition in RM9 medium.

ORGANISMS	INITIAL CELL DENSITY PER ML. (IN 40 ML.)	POL-P in MG. PER LITER			FINAL CELL DENSITY PER ML.
		0 HOUR	18 HOUR	24 HOUR	
(1) <u>P. fluorescens</u>	2×10^6	0.99	0.50	0.26	3.7×10^7
(2) <u>Cl. tetanomorphum</u>	1×10^5	1.49	1.49	1.38	2.3×10^4
(3) Mixed culture (1:1) of (1) and (2)	1.05×10^6	0.88	0.30	0.16	4.4×10^7 (P. fluorescens) 2×10^5 (Cl. tetanomorphum)
(4) The mixed culture (3) plus 1.0 ml. Bard-Parker	1.05×10^6	0.86	0.86	-	0
(5) Medium Control	0	0.82	0.92	0.68	0



of the nutrients by soils does occur, phosphate analyses were made in RM9 medium (1.0 mg. $\text{PO}_4\text{-P}$ per liter) to which various amounts of soil suspension had been added. Table No. 20 shows the results. The residual phosphate in solution was inversely related to the amount of soil suspension added, indicating an uptake of phosphate by soil particles. There was even lower phosphate after longer contact with the soil. To lessen this adsorption problem, the cell inoculum extracted from a soil sample should not be contaminated with large amounts of soil particles. To accomplish this, various filter systems were selected and tested for their capability to retain soil particles while permitting microbial cells to pass through. Ten grams of air-dried soil were extracted with 50 ml. sterile saline and 10-ml. aliquots were filtered through the testing filters. The respective filtrate samples and an untreated control sample were analyzed for microbial count on Nutrient Agar. The result is shown in Table No. 21. The number of microbial cells in the original soil was 4.9×10^5 per gram. The best filter system, from the standpoint of microbial filterability, was the Millipore filter pad and the poorest one was the fritted glass filter having "M" pore size.

b. Unfiltered Soil Samples as Inocula

The untreated soil suspension and each filtrate were then added to the RM9 medium at a ratio of 1:100, 5:100, and 10:100 to evaluate the non-metabolic adsorption of the phosphate by the tested samples. immediately after mixing and after standing for three hours, the medium was filtered again through an ultrafine, fritted glass filter and the remaining phosphate in the filtrate was analyzed. The result is shown in Table No. 22. In spite of previous filtration, all the samples

Table No. 20 - Phosphate uptake by soil particles.

<u>SYSTEM</u> <u>SOIL SUSPENSION" + RMQ</u>	<u>CORRECTION FACTOR ON DILUTION</u>	<u>PHOSPHATE ANALYSES (MG. PER LITER)</u>			
		<u>AT 0 MINUTE</u>		<u>AT 45 MINUTES</u>	
		<u>ACTUAL VALUE</u>	<u>CORRECTED VALUE</u>	<u>ACTUAL VALUE</u>	<u>CORRECTED VALUE</u>
0 ml. + 10 ml.	1.00	0.90	0.90	0.90	0.90
0.1 ml. + 9.9 ml.	1.01	0.98	0.99	0.94	0.95
0.5 ml. + 9.5 ml.	1.05	0.81	0.85	0.75	0.79
1.0 ml. + 9.0 ml.	1.11	0.60	0.67	0.55	0.61

* Soil suspension consists of 13 grams lateritic soil plus 100 ml. sterile saline.

Table No. 21 - Microbial count in soil suspension and soil filtrates* ■

<u>SYSTEMS</u>	<u>MICROBIAL COUNT</u> per ml.
1. Untreated	9.7×10^4
2. Whatman No. 1 filter paper	3.5×10^3
3. Lens paper (20 Layers)	2.5×10^3
4. Millipore filter pad	1×10^4
5. Fritted glass filter (M)	1×10^3

* Soil suspension was prepared by mixing 10 g. air-dried lateritic soil with 50 ml. sterile saline. Ten-ml. aliquots were filtered for preparing filtrate ■

Table No. 22 - Effect of soil particle and soil filtrate on phosphate contents in the RM9 medium.

TREATMENT	SYSTEM		CORRECTION FACTOR ON DILUTION	PHOSPHATE ANALYSIS (MG. PER LITER)					
	SAMPLES ml.	+ RM9 ml.		IMMEDIATELY		AFTER 3 HOURS		ACTUAL VALUE	CORRECTED VALUE
				ACTUAL VALUE	CORRECTED VALUE	ACTUAL VALUE	CORRECTED VALUE		
1. Unfiltered	0.1	+	9.9	1.01	0.80	0.81	0.81	0.82	
	0.5	+	9.5	1.05	0.56	0.59	0.53	0.56	
	1.0	+	9.0	1.11	0.36	0.40	0.32	0.35	
2 Lens paper (20 layers)	0.1	+	9.9	1.01	0.76	0.77	0.57	0.58	
	0.5	+	9.5	1.05	0.71	0.75	0.57	0.60	
	1.0	+	9.0	1.11	0.68	0.75	0.52	0.57	
3 Whatman #1	0.1	+	9.9	1.01	0.66	0.67	0.57	0.58	
	0.5	+	9.5	1.05	0.62	0.65	0.55	0.58	
	1.0	+	9.0	1.11	0.58	0.64	0.55	0.61	
4 Filter pad (millipore)	0.1	+	9.9	1.01	0.64	0.65	0.55	0.56	
	0.5	+	9.5	1.05	0.64	0.67	0.57	0.60	
	1.0	+	9.0	1.11	0.60	0.66	0.52	0.57	
5. Fritted glass filter (M)	0.1	+	9.9	1.01	0.90	0.91	0.84	0.85	
	0.5	+	9.5	1.05	0.64	0.67	0.60	0.63	
	1.0	+	9.0	1.11	0.60	0.66	0.54	0.60	
6 Medium control	0	+	10.0	1.0	0.94	0.94	0.94	0.94	



showed some degree of phosphate adsorption. With the exception of the 1:100 ratio of unfiltered sample, the phosphate adsorption in other samples was time-dependent. This indicated that perhaps the clay fraction having sizes of less than 2 μ had come through all the filter systems and thus participated in phosphate adsorption and so decreased the soluble phosphate in the medium. Based upon present data, it seems that the best inoculum sample from field soil should be the unfiltered 1:5 soil suspension, which, when added to RM9 medium as inoculum, should be applied at 1:100 ratio. This experiment clearly demonstrated that the filter systems suggested above for removal of soil particles are not necessary as long as the unfiltered soil suspension is added to the medium at a 1:100 ratio., Unnecessary loss of cells by the filtration can thus be avoided.

c. Experiment

Two representative soils, silty clay loam and lateritic clay soils, were selected to provide inocula for these tests. The former represents rich loam soil; the latter, poor clay soil.

For the preparation of the inoculum, a 10 g. fresh air-dried soil sample was added to 50 ml. of sterile saline solution. The suspension was stirred with a magnetic stirrer for 10 minutes, and then allowed to sit for five minutes to permit large soil particles to settle. The supernatant was used as the inoculum. From the inoculum, 0.4, 1.0, and 2.0 ml. aliquots were added to 39.6, 39.0, and 38.0 ml., respectively, of RM9 medium. A similar setup for poison control was also included. All the cultures were incubated statically at 26° C., and analyses for ATP and phosphate were made at 0 time and after 24 hours incubation. Cell number was estimated by



spread plate method, using Tryptic Soy Agar medium. The results for the silty clay loam soil are shown in Table No. 23.

The phosphate uptake was distinctly shown in experimental cultures where uptake was almost complete in the highest inoculum within 24-hour incubation. There was a positive relationship between both the initial and final cell density and the phosphate uptake after 24-hour incubation. The slight changes in phosphate concentration in both poison controls and media control were probably due to experimental variation.

Similar analyses using the lateritic clay soil sample as the inoculum are shown in Table No. 24. The soil suspension was prepared by mixing 10 g. air-dried soil with 50 ml. sterile saline as described above.

Phosphate analysis also indicated the presence of microbial activities in the experimental cultures. The highest inoculated culture decreased the phosphate from 0.90 at 0 hour to 0.22 mg. per liter at the 24-hour incubation. Very little phosphate change was observed in the poison and medium control samples. No adsorption of $\text{PO}_4\text{-P}$ by soil particles was apparent.

By comparing the results of experimental and poison controls for phosphate assay systems, both soils clearly demonstrated the feasibility of providing adequate inocula for detecting microbial growth in RM9 medium.

Table No. 23 - $\text{PO}_4\text{-P}$ uptake and ATP analysis of RM9 media inoculated with silty clay loam soil suspension and incubated at 26°C . statically.

SYSTEMS	SOIL I	CULJUM + RM9 ml.	0 HOUR				HOUR			
			CELL DENSITY per ml.	ATP (NA.)	$\text{PO}_4\text{-P}^*$ (MG/L)		CELL DENSITY per ml.	ATP (NA.)	$\text{PO}_4\text{-P}^*$ (MG/L)	
Experimental	0.4	+ 39.6	3.3×10^3	0.25	0.81		1.6×10^7	33.0	0.34	
	1.0	+ 39.0	8.3×10^3	0.26	0.92		2.0×10^8	29.0	0.12	
	2.0	+ 38.0	1.7×10^4	1.25	0.90		3.0×10^8	25.5	0.03	
Poison Control**	0.4	+ 39.6	3.3×10^3	0.17	0.91		1.4×10^2	0.04	0.95	
	1.0	+ 39.0	8.3×10^3	0.30	0.88		3.5×10^2	0.09	0.69	
	2.0	+ 38.0	1.7×10^4	0.30	0.82		9.0×10^2	0.41	0.88	
Media Control	0.0	+ 40.0	0	0.10	0.90		0	0.03	0.86	

* Phosphate concentration in RM9 diluted by soil inoculum was adjusted by calculation.

** Bard-Parker control, 0.2 ml. per 40 ml.

- 4 . 24 - $\text{PO}_4\text{-P}$ uptake and ATP analysis of RM9 medium inoculated with lateritic clay soil suspension and incubated at 26°C . statically.

SYSTEMS	SOIL INOCULUM + RM9 ml.	0 HOUR			24 HOUR		
		CELL DENSITY per ml.	ATP (NA.)	$\text{PO}_4\text{-P}^*$ (MG/L)	CELL DENSITY per ml.	ATP (NA.)	$\text{PO}_4\text{-P}^*$ (MG/L)
Experimental	0.4 + 39.6	12	0.03	0.94	2.7×10^5	0.75	0.83
	1.0 + 39.0	30	0.08	0.90	5.7×10^6	12.5	0.66
	2.0 + 38.0	60	0.05	0.90	6.3×10^6	14.5	0.22
Poison Control**	0.4 + 39.6	12	0.00	0.90	0	0.00	0.87
	1.0 + 39.0	30	0.05	0.82	0	0.00	0.74
	2.0 + 38.0	60	0.02	0.90	0	0.0	0.96
Media Control	0 0 + 40.0	0	0	1.0	0	0.0	0.89

* Phosphate concentration in RM9 diluted by soil inoculum adjusted by calculation.

** Bard-Parker control, 0.3 ml. per 40 ml.



III. METABOLIC UPTAKE OF SULFATE

Sulfur is essential in terrestrial biochemistry. Its ability to function as an analogue of phosphorus in the formation of high energy bonds adds strongly to its potential use in an alien biochemistry. Hence, the sulfate uptake experiment offers an attractive method to monitor the metabolism of either type of life.

Sulfate is generally taken up by microorganisms and then converted to sulfur-containing amino acids such as cystine, cysteine or methionine (11). Some anaerobic microorganisms such as Desulfovibrio however, reduce SO_4^{2-} to adenosine 5'-phosphosulfate (APS). The end product of this reduction generally results in formation of H_2S (12). This group of microorganisms is anaerobic and tolerate extreme terrestrial conditions of heat, cold, salinity and pressure. Therefore, in exploring the area of exobiology, this group of organisms is worth investigating.

Regardless of the metabolic pathway of sulfate in terrestrial or extraterrestrial microorganisms, if it is utilized, it must be taken up from the environment. Accordingly, if a system can be devised to monitor the accumulation of radioactivity in cells, the use of ^{35}S offers a ready means for such monitoring. Unlike ^{32}P , the ^{35}S isotope has a half-life sufficiently long to permit its use in a planetary experiment.

A. Analytical Method

Isotopically labeled sulfur in the form of sulfuric acid as received from New England Nuclear Corporation was carrier-free and had an activity of 50 millicuries (mc.) per ml. A volume of 0.05 ml.



was diluted to 100 ml. with sterile distilled water. This is called the stock solution. Unless otherwise specified, 0.4 ml. of stock solution was added to 40 ml. RM9 medium to make RM9- $^{35}\text{SO}_4$ for sulfate uptake studies.

The inoculum was washed, transferred to 40 ml. of a medium containing $^{35}\text{SO}_4$ -S and incubated. After various lengths of incubation intervals, one-ml. aliquots of the culture were removed, filtered through 0.45 μ . HA membrane filter and rinsed with 2 ml. of sterile saline to remove interstitial $^{35}\text{SO}_4$ -S. The cells on the membrane were then dried under an infrared lamp for at least 15 minutes and their radioactivity measured by a Nuclear Chicago D-47 gas-flow counter.

Labeled sulfur in thioglycollic acid was also tested for the sulfur uptake study. This compound was found to be necessary for growing anaerobes and is one of the ingredients in the RM9 medium. The compound when received from New England Nuclear Corporation, had a specific activity of 1.0 mc. per mM. A total volume of 0.064 ml. was diluted 1:1000 with sterile distilled water. One ml. of the diluted solution was added to 39 ml. of M9 medium. Sulfur uptake followed the $^{35}\text{SO}_4$ -S uptake method.

Radioactive sulfur has a half-life of 87.1 days. In eight month's time the radioactivity will be reduced to about 19% of the original activity. Therefore, a 50 mc. solution will have 9.5 mc. after an eight-month period. The decrease, however, will not invalidate the proposed sulfate uptake technique for the mission of life detection on Mars.



B. Experimental

1. Development of Medium

As discussed above, (Section 11, B, 1), the M9 medium was initially used for testing sulfate uptake as it was for the phosphate uptake study. However, since the MgSO_4 in M9 medium was rather high (0.2 g. per liter), the non-labeled sulfate ion could dilute the $^{35}\text{SO}_4\text{-S}$ and cause less pronounced results of $^{35}\text{SO}_4\text{-S}$ uptake. To remedy this, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was reduced to 0.08 g. per liter.. The RM9 (Table 4) was finally adopted also for sulfate uptake studies. This medium has been proven as good for sulfate uptake as for phosphate uptake studies.

2. Poison

The poisons for sulfate uptake studies were Bard-Parker, 4% HgCl_2 solution and 70% perchloric acid solution. From three-tenths to 1 ml. poison solution per 40 ml. medium have proved to be quite effective.

3. Theoretical Consideration of Sulfate Uptake

The sulfur content of average bacteria is 10 mg. per one gram dry cell weight, or 1%. Since the dry weight of 10^7 cells is approximately 6.7 $\mu\text{g.}$ (9), the sulfur content in 10^7 cells will be 0.067 $\mu\text{g.}$ The RM9 medium contains 0.08 gram of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per liter or 10 mg. $\text{SO}_4\text{-S}$ per liter. This value is equivalent to 10 $\mu\text{g.}$ per ml. To grow 10^7 cells in one ml. , requires only 0.067 $\mu\text{g.}$ of $\text{SO}_4\text{-S}$, therefore 10 $\mu\text{g.}$ $\text{SO}_4\text{-S}$ per ml. is sufficient to support the growth of more than 10^7 cells per ml. of microorganisms.



4. Pure Culture Studies

a. M9 or Modified M9 Medium

(1) Sulfate Uptake at 37° C. Static Conditions

Radioactive sulfur was supplied in sulfate form for this uptake study. The activity level was adjusted to approximately 6×10^4 cpm per ml. of the culture.

The experimental arrangements and results on uptake by P. fluorescens and E. coli are shown in Table No. 25. Inocula of each organism were grown in M9 (1.0 mg. $\text{PO}_4\text{-P}$ per liter). plus 0.2% glucose. The test medium was composed of 1.8 mg. per liter of Na-thioglycollate in addition to the ingredients of M9 medium. Uptake by each species was indicated immediately upon exposure, but was established with certainty by the third hour.. Despite higher initial cell density in the testing media for P. fluorescens than for E. coli, the uptake by the latter was slightly higher than the former.

The M9 medium was further modified by reducing sulfate concentration ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) from 0.2 g. to 0.004 g. per liter. With the presence of $^{35}\text{SO}_4\text{-S}$ the medium was initially monitored at 2×10^4 cpm per ml. An estimated 5×10^4 cells/ml. of E. coli were inoculated into 40 ml. of M9 medium containing 0.125% glucose. The medium thus modified is referred to as M9TG. Control cultures were treated with Bard-Parker germicide. The complete experimental design and results are shown in Table No. 26. The cultures were incubated statically at 37° C, and, at the designated times, one-ml. samples were filtered, washed with 2.5 ml. of saline solution, and the filters and cells dried and counted. The results showed a rapid, but low level initial uptake of sulfate in the unpoisoned cultures (see Table No. 26).

Table No. 25 - $^{35}\text{SO}_4$ -S uptake by aerobic Pseudomonas fluorescens
and facultative anaerobic Escherichia coli
under static condition at 37°C .

TEST ORGANISMS TEST ORGANISMS	INITIAL CELL DENSITY PER ML. (10 ML. CULTURE)	CPM OF FILTER		CPM OF FILTER PLUS CELLS		
		LESS CELLS		0 hr.	3 hr.	24 hr.
		0 hr.	24 hr.			
<u>P. fluorescens</u>	5×10^4	12	10	23	108	1182
<u>E. coli</u>	3×10^4	12	10	34	139	1544

Modified M9: per liter: K_2HPO_4 , 5.0 mg.; NH_4NO_3 , 0.2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0039 g.;
NaCl, 0.1 g.; soil extract, 100 ml. 3 (per 900 ml.) glucose, 1.25 g.;
Na-thioglycollate, 0.0018 g.

Table No. 26 - $^{35}\text{SO}_4$ -S uptake by ~~E. coli~~ under 37°C .
static condition, Initial cell density,
 5×10^4 per ml.

TREATMENT (40ML. MEDIUM)	CPM OF FILTER LESS CELLS 0 hr.	CPM OF FILTERS PLUS CELLS			
		0 hr.	3 hr.	5 hr.	24 hr.
1. M9	9	29	52	70	3000
2. M9 + 0.1 ml. B-P	9	32	13	24	7
3. M9 + 3.0 ml. B-P	6	38	7	7	15
4. M9TG	7	35	38	30	401
5. M9TG + 0.1 ml. B-P	4	20	24	3	1
6. MgTG + 3.0 ml. B-P	3	21	3	44	19

M9: per liter: K_2HPO_4 , 5.0 mg.; NH_4NO_3 , 0.2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.004 g.;
 NaCl , 0.1 g.; soil extract, 100 ml.; glucose, 1.25 g.

M9TG: M9 medium plus 0.1% Na-thioglycollate

B-P: Bard-Parker germicide

The uptake increased materially sometime after the fifth hour.

There was less $^{35}\text{SO}_4$ -S uptake in the M9TG medium than in the M9.

This was apparently because E. coli prefers thioglycollate-S to SO_4 -S. The poisoned cultures showed lower count levels than the unpoisoned ones at all time intervals and the difference became unequivocal sometime after the fifth hour.

(2) Sulfate Uptake at Room Temperature: Aerated Condition

A further test was made on uptake of $^{35}\text{SO}_4$ -S by P. fluorescens under room temperature and aerated condition (20 ml. per minute per 40 ml. culture), with and without Na-thioglycollate and with different glucose levels to verify previous results and also to investigate possible antagonism of Na-thioglycollate to $^{35}\text{SO}_4$ -S uptake, Table No. 27 shows the results. Na-thioglycollate did decrease the $^{35}\text{SO}_4$ -S uptake. There were approximately three times more sulfate uptake at 24 hours without thioglycollate than with thioglycollate. The glucose concentration played an important role in increasing sulfur uptake as was also shown in the phosphate uptake study. Highest uptake was shown in the culture containing the highest glucose concentration. This is consistent in cultures both with and without thioglycollate. The uptake of radioactive sulfur by the poisoned cultures probably reflected the irreversible adsorption of the $^{35}\text{SO}_4$ -S by dead cells because the plate count did not show any growth in these cultures.

(3) Sulfate Uptake of an Anaerobe Under Static and N_2 Agitation

A similar study using M9TG medium was run on C1. tetanomorphum, with the objective of learning the effect of glucose concentration on sulfate uptake of this anaerobic organism. The experimental design

Table No. 27 - $^{35}\text{SO}_4$ -S uptake of *Pseudomonas fluorescens*
under room temperature aerobic condition
at different glucose concentrations with
and without thioglycollate. Initial cell
density, 1×10^4 per ml. of 40 ml. culture.

TREATMENTS	CPM OF FILTER LESS CELLS		CPM OF FILTER PLUS CELLS			PLATE COUNTS AT 24 HR. ml.
	0 hr.	24 hr.	0 hr.	3 hr.	24 hr.	
1. M9 + 0.2% glucose	28	30	78	231	2591	1×10^8
2. M9 + 0.02% glucose	50	30	64	124	635	7.8×10^7
3. M9 + 0.002% glucose	27	32	82	100	448	5.4×10^7
4. M9 + 0.2% glucose + 1.0 ml. B-P	33	41	8%	8	91	0
5. M9TG + 0.2% glucose	33	27	88	317	778	1.7×10^8
6. M9TG + 0.02% glucose	9	22	84	112	520	8.9×10^7
7. M9TG + 0.002% glucose	37	32	82	22	265	7.6×10^7
8. M9TG + 0.2% glucose + 1.0 ml. B-P	33	35	89	39	106	0

M9: per liter: K_2HPO_4 , 5.0 mg.; NH_4NO_3 , 0.2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.08 g.; NaCl, 0.1 g.
soil extract 100 ml. (per 900 ml.); ^{35}S glucose concentration at indicated concentration.

MgTG: M9 medium plus 0.1% Ma-thioglycollate.

B-P: Bard-Parker.



and the results are summarized in Table No. 28. In these tests, the concentration of glucose was varied (0.2%, 0.02% and 0.002%) and the concentration of ordinary sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) was reduced from 0.2 g. per liter to 0.08 g. per liter. The amount of Na-thioglycollate added was 1.0 g. per liter of medium. One culture was poisoned with Bard-Parker germicide as a control. The results showed that, even at the lowest concentration, glucose was not limiting until after the third hour, and that it did not become limiting in proportion to its concentration.

One additional group of tests was run to determine if agitation will increase sulfate uptake by cultures of C1. tetanmorphum. The experimental design and results of these tests are shown in Table No. 29. Cultures were grown in M9TG and in M9 with various concentrations of glucose. In both cases, the Concentration of stable sulfate ions was decreased to foster increased uptake of $^{35}\text{SO}_4\text{-S}$. All the cultures were agitated with N_2 gas bubbled at a rate of 20 ml. of gas per min. per 40 ml. of medium. There was no appreciable difference between cultures grown on M9 and on M9TG, but neither medium produced the level of response obtained in the static test.

(4) ^{35}S -thioglycollate Uptake Study

^{35}S -thioglycollate (37 μg . per 40 ml. medium) was added to two test media for sulfur uptake study. One is M9 less MgSO_4 , but with non-labeled Na-thioglycollate at the concentration of 1.4 mg. per 40 ml. medium. The other medium is M9 with non-labeled $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ at the concentration of 3 mg. per 40 ml. medium. This second medium did not have non-labeled Na-thioglycollate. Each medium, in addition, was incorporated with 0.2, 0.02, and 0.002% of glucose to see if the

Table No. 28 - $^{35}\text{SO}_4$ -S uptake by Clostridium tetanomorphum under 37°C . static anaerobic condition at different glucose concentrations. Initial cell density, 1×10^4 per ml.

TREATMENT (40 ML. MEDIUM)	GM OF FILTER LESS CELLS	GM OF FILTER PLUS CELLS				
	0 time	0 time	1 hr.	3 hr.	5 hr.	24 hr.
1. M9TG + 0.2% glucose	46	95	1-15	126	138	508
2. MgTG + 0.02% glucose	72	99	95	103	115	275
3. M9TG + 0.002% glucose	73	98	98	108	71	130
4. MgTG + 0.2% glucose + 0.1 ml. B-P	56	80	53	33	13	15

M9TG: per liter: K_2HPO_4 , 5.0 mg.; NH_4NO_3 , 0.2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.08 g.; NaCl, 0.1 g.; soil extract, 100 ml.; Na-thioglycollate, 1.0 g.; glucose at indicated concentrations.

B-P: Bard-Parker germicide.

Table No. 29 - Uptake of $^{35}\text{SO}_4$ -S by *Clostridium tetanomorphum* at 37°C . under agitation by N_2 gas (20 ml/min/40 ml) at different glucose concentrations. Initial cell density, 10^4 per ml.

TREATMENT (40 ML MEDIUM)	GM OF FILTER LESS CELLS	GM OF FILTER PLUS CELLS				
	0 time	0 hr.	1 hr.	3 hr.	5 hr.	24 hr.
1. M9 + 0.2% glucose	56	73	105	156	184	127
2. M9 + 0.02% glucose	58	88	103	122	157	32
3. M9 + 0.002% glucose	52	85	107	118	155	59
4. M9 + 0.2% glucose + 0.1 ml. B-P	55	79	64	53	15	20
5. M9TG + 0.2% glucose	39	108	123	166	219	66
6. M9TG + 0.02% glucose	32	97	103	138	196	57
7. M9TG + 0.002% glucose	57	102	103	130	176	42
8. M9TG + 0.2% glucose + 0.1 ml. B-P	34	76	61	56	15	42

M9: per liter of KH_2PO_4 , 5.0 mg.; NH_4NO_3 , 0.2 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.08 g.; NaCl, 0.1 g.; soil extract, 100 ml.; glucose at indicated concentrations.

M9TG: M9 medium plus 0.1% Na-thioglycollate.

B-P: Bard-Parker .



glucose concentration exerts any influence on the sulfur uptake.

Twenty-four hour culture of E. coli was washed and the cells were then inoculated into 40 ml. medium at the concentration of 1×10^4 cells per ml. Poison controls were also included. The result is shown in Table No. 30. No significant sulfur uptake has been shown in this study indicating that thioglycollate is not a suitable substrate to provide sulfur for E. coli. Its main function in the medium is perhaps merely to decrease O-R potential to create a favorable condition for the growth of anaerobes.

b. RM9 Medium

(1) Sulfate Uptake at Room Temperature; Static Condition

P. fluorescens, E. coli, and C1. tetanomorphum were selected for testing the radioactive sulfate uptake in the RM9 medium. Cell inocula from each organism in 40 ml. culture were respectively, 0.6×10^4 , 1.8×10^4 , and 1.8×10^4 cells per ml. Arrangement of treatment and results of analysis are shown in Table No. 31. The RM9 medium was extremely good for sulfate uptake since there was noticeable sulfate uptake even at five-hour incubation. The cpm for P. fluorescens, E. coli, and C1. tetanomorphum were , respectively, 521, 2332, and 2554 at five hours, and 3919, 4819, and 3368 at 24 hours. The poisoned controls were about 50 cpm and the medium controls were much less at 24 hours.

(2) Thioglycollate Effect on Sulfate Uptake and Growth of an Anaerobe

The anaerobic organisms generally are much slower in growth when compared with the aerobic or facultative anaerobic organisms. Previous experiments on sulfate uptake have been limited to 24-hour

Table No. 30 - Sulfur uptake by E. coli in M9 medium plus labeled ^{35}S -thioglycollate at 37°C and static incubation.

	GM OF CELLS PLUS FILTER				CELL NO./ML.
	0	2 hr.	4 hr.	<u>24 hr.</u>	24 hr.
<u>Medium Controls</u>					
MA + 0.2% glucose	10	13	8	13	0
MB + 0.2% glucose	17	20	12	12	0
<u>Experimental</u>					
MA + 0.2% glucose	33	22	35	27	2.5×10^8
MA + 0.02% glucose	29	17	21	25	1.4×10^8
MA + 0.002% glucose	24	22	23	33	7.4×10^7
MB + 0.2% glucose	42	29	70	123	7.5×10^8
MB + 0.02% glucose	19	29	49	107	2.9×10^8
MB + 0.002% glucose	30	22	34	87	1.1×10^8
<u>Poison Controls</u>					
MA + 0.2% glucose + BP	64	19	15	19	0
MB + 0.2% glucose + BP	23	45	21	14	0

MA: M9 less MgSO_4 plus thioglycollate (1.4 mg. per 40 ml.) and ^{35}S -thioglycollate (37 μg . per 40 ml.).

MB: M9 with $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (3 mg. per 40 ml.) and ^{35}S -thioglycollate (37 μg . per 40 ml.).

BP: Bard-Parker, applied at 0.2 ml. per 40 ml. medium.

Table No. 31 - ³⁵SO₄-S uptake by Pseudomonas fluorescens, Escherichia coli, and Clostridium tetanomorphum at room temperature (26° C.), static condition in revised M9 medium*.

ORGANISMS	INITIAL CELL DENSITY PER ML. (IN 40 ML.)	TREATMENTS (IN 40 ML.)	CPM OF FILTER PLUS CELLS**			PLATE COUNT AT 24 HOURS per/ml
			0 hr.	5 hr.	24 hr.	
<u>P. fluorescens</u>	0.6 x 10 ⁴	1. Experimental	33	521	3919	4 x 10 ⁷
		2. + 0.1 ml. Bard-Parker	60	238	40	0
		3. + 0.1% HgCl ₂	50	125	49	0
<u>E. coli</u>	1.8 x 10 ⁴	1. Experimental	44	2332	4819	4.4 x 10 ⁸
		2. + 0.1 ml. Bard-Parker	38	292	91	0
		3. + 0.1% HgCl ₂	89	133	46	0
<u>C. tetanomorphum</u>	1.0 x 10 ⁴	1. Experimental	40	2554	3368	5.5 x 10 ⁷
		2. + 0.1 ml. Bard-Parker	52	293	49	0
		3. + 0.1% HgCl ₂	54	97	39	0

- * RM9: per liter: K₂HPO₄, 5.0 mg.; NH₄NO₃, 0.2 g.; MgSO₄·7H₂O, 0.08 g.; NaCl, 0.1 g.; soil extract, 100 ml. (per 900 ml.); glucose, 0.2 g.; Na-thioglycollate, 0.002 g.; Tris, 6.0 g., pH 7.0.
- * All the cpm have been corrected for background count.

studies. The following experiment was to find the peak of growth and sulfate uptake of Cl. tetanomorphum at prolonged incubation under the influence of various thioglycollate concentrations.

The experimental setup and results are shown in Table No. 32. It is interesting to note that thioglycollate at higher concentrations did hasten the growth and uptake of sulfate but sulfate leaching occurred rapidly after 48 hours of incubation. Although sulfate uptake at 24 hours was lower for the thioglycollate at 0.002 gram per liter (a normal concentration in RM9), the value was considerably higher than the poisoned control. Prolonged incubation of this sample showed a steady uptake of sulfate, without leaching, up to 144 hours.

(5) Mixed Culture Studies

The P. fluorescens and Cl. tetanomorphum were grown separately on Tryptic Soy Broth and Fluid Thioglycollate media, respectively. They were washed and resuspended in saline. Individual cultures and a mix of both cultures at 1:1 volume ratio were inoculated into 40 ml. of RM9 medium. Initial cell density and the result of sulfate uptake are shown in Table No. 33. The highest uptake was demonstrated by the Pseudomonas culture (856 cpm) while the lowest was from the Clostridium culture (472 cpm). The value for the mixed culture was intermediate between these two pure cultures. The individual species in the mixed culture were estimated by growing Clostridium on thioglycollate agar under anaerobic conditions and Pseudomonas on Tryptic Soy Agar under aerobic conditions. The latter cannot grow under anaerobic conditions and the former cannot survive under aerobic conditions. The number of each species in the mixed culture was comparable to the number in the respective pure cultures.

4 No. 32 - Effect of thioglycollate concentration and length of incubation on growth and $^{35}\text{SO}_4$ -S uptake of Clostridium tetanomorphum.

	TREATMENTS	0 HR.		24 HR.		48 HR.		72 HR.		144 HR. CPM
		CELL DENSITY*	CPM**	CELL DENSITY	CPM	CELL DENSITY	CPM	CELL DENSITY	CPM	
1	RM9 medi control	0	101	0	99	0	104	0	74	103
2	RM9 + thioglycollate ^a (0.002 g/l)	3.8×10^4	111	1.7×10^4	474	3.5×10^5	13	7×1	3117	3272
3	RM9 + thi glycollate ^a (0.05 g/l)	3.8×10^7	103	1.5×10^4	9	5.0×10^6	2417	5.6×10^7	1625	738
4	RM9 + thioglycollate ^a (0.01 g/l)	3.6×10^4	96	1.9×10^5	66	4.0×10^6	2886	1.1×10^7	346	159
5	RM9 + thioglyc late ^a (0.05 g/l)	5.6×10^4	135	7.4×10^5	1153	5×10^6	3360	-	768	305
6	RM9 + thioglycollate (0.1 g/l)	3.8×10^4	204	1.2×10^6	2303	6.4×10^7	5412	-	319	350
7	RM9 + thioglycollate (0.1 g/l) + 0.3 ml. Bard-Parker	3.8×10^4	121	0	132	0	149	0	157	305
8	RM9 + thioglycollate (0.1 g/l) + 0.3 ml. 70% perchloric acid	3.8×10^4	79	0	140	0	136	0	145	240

* Cell density: number of cells per ml. in 40 ml. culture.

** CPM: cpm of $^{35}\text{SO}_4$ -S in cells plus filter.

Table No. 33 - $^{35}\text{SO}_4$ -S uptake by pure and mixed cultures of Pseudomonas fluorescens and Clostridium tetanomorphum at room temperature (26° C.), static condition in revised M9 medium.

ORGANISMS	INITIAL CELL DENSITY/ML (IN 40 ML.)	CPM OF FILTER PLUS CELLS			FINAL CELL DENSITY/ML
		0 HR.	18 HR.	24 HR.	
(1) <u>P. fluorescens</u>	2×10^6	61	180	856	7.4×10^7
(2) <u>Cl. tetanomorphum</u>	1×10^5	59	114	472	8×10^5
(3) Mixed culture (1:1) of (1) and (2)	1.05×10^6	158	168	551	5.8×10^7 (<u>P. fluor.</u>) 3×10^5 (<u>Cl. tet.</u>)
(4) The mixed culture (3) plus 1.0 ml. Bard-Parker	1.05×10^6	15	37	157	0
(5) Medium control	0	8	15	10	0

(6) Soil Sample Studies

Microbial activities in sulfate uptake using two soil samples as inocula were tested. Two soil samples were selected, i.e., silty **clay** loam and lateritic clay soils. The soil suspensions were prepared according to the procedure described in Section II, B, 6. Various volumes of inoculum were added to the 40 ml. RM9. The experimental setup and results are shown in Table No. 34 for the silty clay loam soil and in Table No. 35, for the lateritic clay soil. Sulfate uptake **was** clearly shown in experimental samples inoculated with both soils. The ratios between the experimental samples and the poison control in sulfate uptake were 10 or 30 to 1, depending upon the initial cell density in the soil samples. A positive relation between initial cell density and sulfate uptake was noted in the clay soil experiment (Table No. 35).

IV. ENZYMATIC ASSAY OF MICROBIAL ATP

This life detection experiment (10,11,12) is based upon the sensitivity and specificity of the firefly lantern bioluminescent system for ATP and upon the ubiquity of ATP in all known cellular material. The bioluminescent reactants of the firefly lantern - luciferase, luciferin, magnesium, and oxygen - are readily extracted in usable **form**. Additional luciferin, which can be synthesized, is added to improve sensitivity. When ATP is injected into this system, light is produced. **The** bioluminescent reaction of the firefly lanterns is

Table No. 34 - $^{35}\text{SO}_4$ -S uptake from RM9 medium inoculated with E. coli ty clay loam soil suspension and incubated at 26°C . statically.

SYSTEMS	INOCULUM + RM9 ml.	0 HOUR		24 HOUR	
		CELL DENSITY/ML IN 40 ML.	CPM OF FILTER PLUS CELLS	CELL DENSITY/ML IN 40 ML.	CPM OF FILTER PLUS CELLS
Experimental	0.4 + 39.6	7.5×10^3	598	3×10^7	1481
Poison Control (+ 0.1 ml. 4% HgCl_2)	0.7 + 39.6	4.5×10^3	51	0	49
Medium Control	0.0 + 39.0	0	30	0	33

Table No. 35 - $^{35}\text{SO}_4$ -S uptake from RM9 medium inoculated with lateritic clay soil suspension and incubated at 26°C . statically.

SYSTEMS	INOCULUM + RM9 ml.	0 HOUR		24 HOUR	
		CELL DENSITY/ML IN 40 ML.	CPM OF FILTER PLUS CELLS	CELL DENSITY/ML IN 40 ML.	CPM OF FILTER PLUS CELLS
Experimental	0.4 + 39.6	88	60	4 x 10^6	449
	1.0 + 39.0	220	107	3 x 10^6	1381
	2.0 + 38.0	440	333	6 x 10^6	2303
Poison Control (+ 0.3 ml. B-P)	0.0 + 40.0	0	24	0	31
	0.4 + 39.6	8	91	0	80
	1.0 + 39.0	0	38	0	149
	2.0 + 38.0	0	10	0	168
Medium Control	0 0 + 40.0	0	37	0	30

specifically for ATP.

A. Analytical Method

Following are the procedures for this assay:

(1) Preparation of Reagents

- a. Tris buffer: 0.05 M Tris, adjusted to pH 7.4 with concentrated HCl, autoclaved, and stored in refrigerator.
- b. MgSO_4 : 0.01 M MgSO_4 prepared in the Tris buffer and autoclaved.
- c. Luciferin: 0.6 mg luciferin per ml. of Tris buffer.
- d. ATP: 1.0 mg. per ml. in Tris buffer **as** stock solution. This is stored frozen and when needed for standard solution, is diluted with Tris buffer.
- e. Saline: 0.85% NaCl in distilled water and autoclaved.
- f. **DMSO**: 90% dimethylsulfoxide in 0.05 M Tris buffer, adjusted to pH 7.4 with HCl and autoclaved.
- g. Luciferase: One gm. acetone powder prepared from active firefly lanterns (Hazleton collection 1966) is extracted with 5 ml. of Tris buffer at 0° C. for 10 min. with occasional stirring. The insoluble portion is removed by centrifugation at 500 x g for 10 min. at 0° C. The supernatant is decanted off and stored at 0° C. The pellet is again extracted with another 5 ml. of Tris buffer in the manner described above. The combined supernatant of approximately 10 ml. (the soluble extract) is then



passed through a Sephadex G-100 column and eluted with Tris buffer. Fractions from the column are collected in 7-10 ml. portions and aliquots from each removed to be assayed for luciferase activity. The most potent ones (approximately 30-40 ml.) were pooled and lyophilized in 2-ml. portions each in tear-drying bulbs. The lyophilized enzyme in bulbs (approximately 20 μ g. each) is stored in a deep freezer (-10° C.) and removed to be reconstituted as required.

(2) Preparation of Reaction Mixture

Lyophilized luciferase is reconstituted by dissolving it in 2.0 ml. of 0.05 M Tris buffer. To this is added 2.0 ml. of MgSO_4 solution and 2.0 ml. of luciferin. The R_x then is filtered through a sterile membrane filter to remove any bacterial contamination.

(3) Extraction of ATP from Microbial Cells

Two ml. of test cultures are filtered through a Millipore HA membrane filter (0.45 μ .) in a Swinnex 13 filter unit. Each filter is previously wetted with 2.0 ml. of 0.05 M Tris buffer and then extracted with 1.0 ml. of 90% DMSO solution at room temperature by injecting the DMSO through the membrane. The membrane will be dissolved and ATP will be released from the cells. To find an optimal ratio between the ATP sample (in 90% DMSO) and the reaction mixture to give best ATP response, both 100 λ



and 10λ standard 10^{-3} μ g. ATP in 90% DMSO were injected into 100λ reaction mixture. The light pulse was detected by a photomultiplier tube and monitored by a strip chart recorder. The peak amplitude of the signal is proportional to the quantity of ATP present. The results shown in Table No. 36 indicate that the 10λ sample gave 60 na, response which was about 80% of the light response of 10λ ATP sample in Tris buffer (76 na.). This indicated that ATP in 90% DMSO can be injected into the reaction mixture to give acceptable light response when the ratio of 1 part ATP in DMSO to 10 parts reaction mixture is used. If a cell culture initially contains 10^7 cells per ml., then number of cells which contribute ATP in a 10λ sample volume will be 6.7×10^4 cells.

(4) Assay Technique

The R_x is placed in a capped, sterile plastic culture tube and allowed to stand at room temperature until the inherent light has decreased. The inherent light is associated with enzyme preparation.' In every assay, the bioluminescent detector is adjusted so that a zero level can be set on the strip chart recorder when no light source is in front of the phototube. A cuvette containing 100λ of R_x is then placed in front of the phototube and the signal noted at this point represents the inherent light of the system. Ten λ of the extracted ATP from microbial cells or

Table No. 36 - Comparison of ATP light response
from 10 λ and 100 λ volumes of **samples**.

<u>TEST ATP SOLUTION</u>	<u>RESPONSE (NA.)</u>
10 ⁻³ pg. in 10 λ Tris	76
10 ⁻³ μ g. in 100 λ Tris	48
10 ⁻³ μ g. in 10 λ DMSO	60
10 ⁻³ pg. in 100 λ DMSO	No response (completely quenched)



standard ATP solution is now injected into the cuvette and the response in nanoamperes (na.) is recorded. Gross response is reported as the total increase from the initially adjusted zero level, including inherent light, while the net response is gross response less inherent light. The net response is used for the ATP index. For quantitation of unknown ATP, at least three net responses of standard ATP solutions ranging from 10^{-3} $\mu\text{g.}$ to 10^{-5} $\mu\text{g.}$, or 10^{-6} μg per 10 λ should be included so that the unknown value can be extrapolated from the standard curve of na. vs. ATP concentration. Figure No. 17 shows a typical calibration curve for the EMR instrument.

(5) Instrument

Most of the measurements of light output made as an integral part of biochemical research during this contract were conducted on a bioluminescent detector on hand at Hazleton Laboratories. A block diagram of the instrument is shown in Figure No. 18. This instrument was manufactured by Electromechanical Research, Inc., (EMR) and is configured to hold a cuvette or 6 mm. x 50 mm. test tube as a reaction chamber. The reaction is initiated by injecting, with a syringe, the ATP sample into a test tube containing the reaction mixture. The instrument employed an oscilloscope calibrated in nanoamperes.

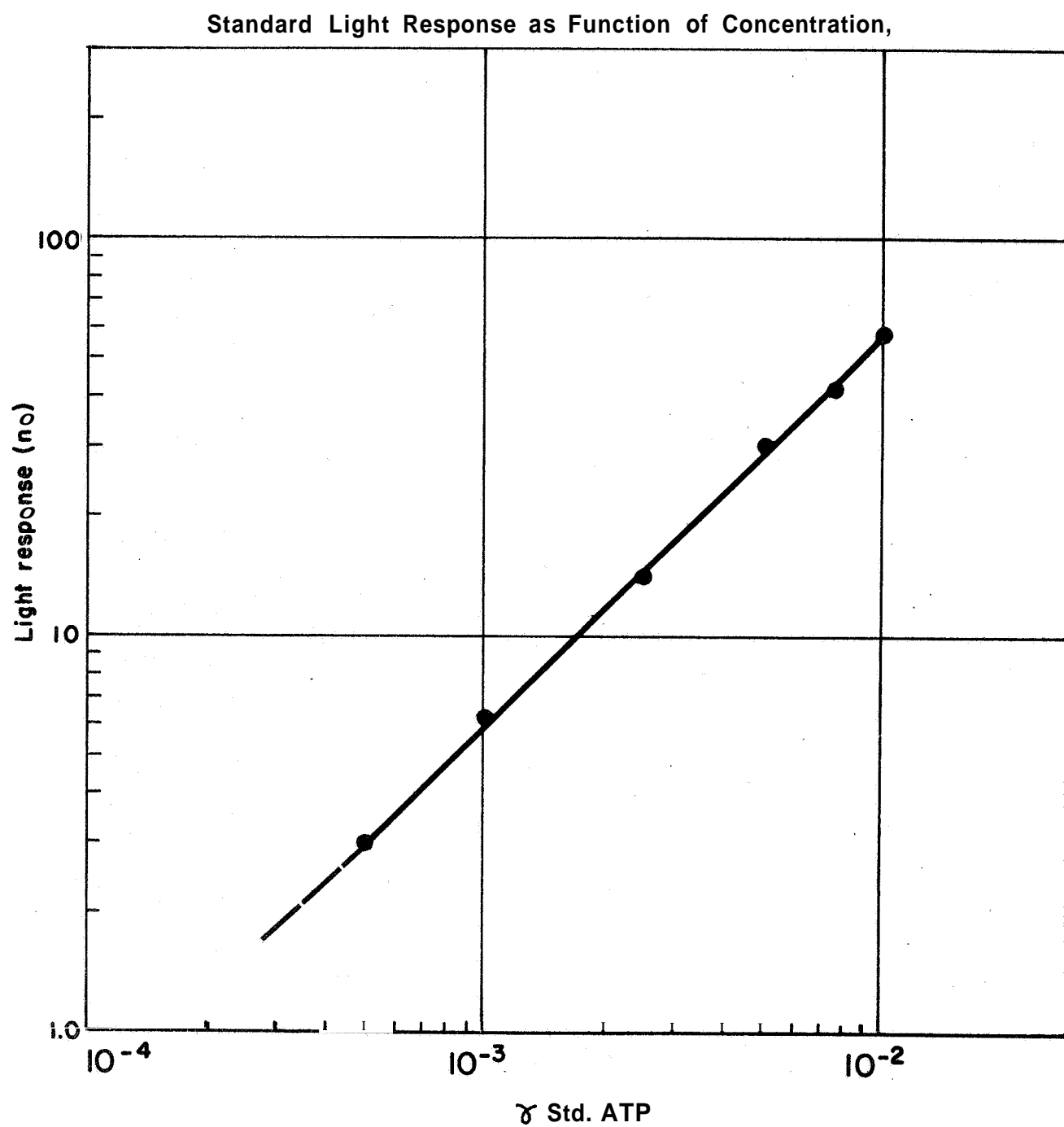


Figure No.17 - Typical calibration curve (EMR instrument)

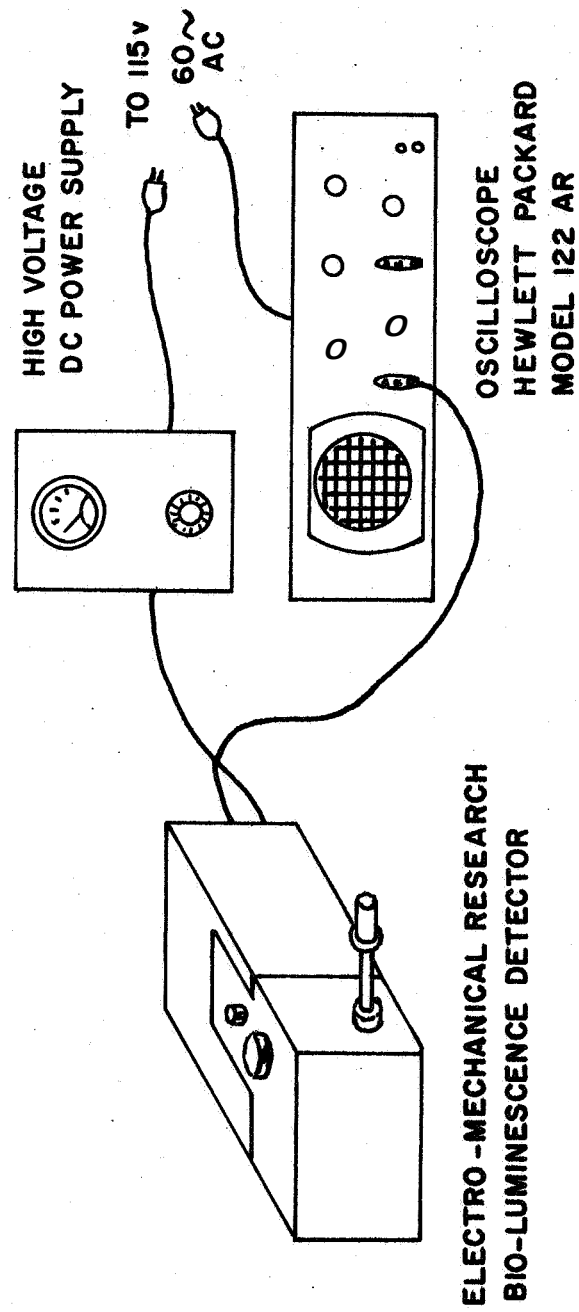


Figure No.18 -EMR BIO-LUMINESCENCE DETECTOR BLOCK DIAGRAM

B. Experimental

1. Medium

Any medium which supports the growth of microorganisms will provide cells to give an ATP response and the ATP response is proportional to the number of cells (10). In this program, the medium for ATP studies was limited to RM9.

2. Poison

Studies on ATP response from cultures treated with various poisons are shown in Table No. 10. The Bard-Parker and 70% perchloric acid at the tested levels were very effective in suppressing the microbial growth as shown by the ATP response. Other poisons tested for phosphate and sulfate are equally applicable to ATP tests.

3. Theoretical Consideration of ATP Production

The average bacterial cells contain 10^{-9} $\mu\text{g.}$ ATP per cell. The larger cells such as yeast and spores of Aspergillus contain respectively, 3×10^{-8} $\mu\text{g.}$ and 2×10^{-7} $\mu\text{g.}$ per cell (10). Assuming ATP content in a cell is 10^{-9} $\mu\text{g.}$, then 10^7 cells will give 10^{-2} $\mu\text{g.}$ ATP which can easily be detected by present ATP assay system.

4. Pure Culture Studies

Pure culture study was made on E. coli. Initial cell density in 40 ml. of RM9 medium was 4.6×10^6 per ml. The result is shown in Table No. 10. Two ml. of cell suspension at zero time were filtered and extracted with 90% DMSO. One-tenth ml. was injected into 100 λ reaction mixture; the response being 1 na. After a 24-hour incubation, ATP determination was made again. This time the response

was 26 na., indicating growth of microorganisms. The plate count at 24 hours showed the cell density was 5.5×10^8 per ml. The medium and strong Bard-Parker poisoned control at 24-hour incubation did not give ATP response.

5. Soil Sample Studies

The soil samples (silty clay loam and lateritic clay soils) used for phosphate uptake studies were also tested for ATP production in RM9 medium. The experimental setup and the results of the test are shown in Tables No. 23 and No. 24.

The results for the silty clay loam soil inoculum are shown in Table No. 23. A drastic increase of ATP from 0 to 24-hour incubation is seen in all three experimental samples. At zero time, ATP responses ranged from 0.25 to 1.25 na., while at 24-hour incubation, the values ranged from 25.5 to 32 na. An inverse relation between the amount of inoculum and ATP content at 24 hours was noted. This appeared to reflect the age of the cultures. At a given length of incubation period, the cultures with the higher inoculum generally would reach the stationary phase faster than the culture with lower inoculum; the latter probably still being at the lag phase. It is believed that cells at the stationary phase contain less ATP than the cells at the lag phase.

The ATP in the poisoned control and in the medium control (less inoculum) was practically nil, indicating that there was no microbial growth.

Similar analyses using a lateritic clay soil sample as the inoculum are shown in Table No. 24. The microbial count of the lateritic clay soil was very low because the initial cell density in the three experimental cultures ranged only from 12 to 60 cells per ml. Despite



this, the ATP yield at 24-hour incubation ranged from 0.75 to 14.5 **na.**, whereas the poisoned and media controls did not give any ATP response, The positive relation between the amount of inoculum or the number of cells and ATP content indicated that all the cells were probably still in the lag phase, since the highest final cell density was only 6.3×10^6 per ml., equivalent to growth at the lag phase.

By comparing the results of the experimental and the poisoned controls for ATP assay systems, both soils clearly demonstrated the feasibility of providing adequate inocula for detecting microbial growth in RM9 medium.

V. GROWTH TEST OF VARIOUS ORGANISMS IN **THE RM9 MEDIUM**

The RM9 medium was tested for growing various microbial species. Each organism was grown first on a standard medium. After washing with saline, the cell suspension was inoculated into 10 ml. RM9 medium in screw cap tubes. All the cultures were maintained at 26°C . under static conditions for 24 to 96 hours. Their growths were observed by relative turbidity. The result is recorded in Table **No. 37**.

Other sets of microorganisms were selected for the growth test in RM9 medium. This time a loopful of each culture was transferred from an agar slant to a screw cap test tube containing 10 ml. RM9 medium. Incubation **was** at 26°C . statically. The results are shown in Table **No. 38**. Quite a number of cells grew within 24 hours; however, many other organisms required longer incubation for visual detection of their growth. The reason for this **delay** may be due to their preadaptation in the stock culture media.

Table No. 37 - Growth of various organisms in ~~RM~~
 medium at 26° C. under static condition.

<u>ORGANISMS</u>	<u>ENERGY SOURCE</u>	<u>C₂ RELATION</u>	<u>GROWTH</u> [*]
<u>Bacillus subtilis</u>	heterotrophic	aerobic	++
<u>Escherichia coli</u>	heterotrophic	facultative anaerobic	f+++
<u>Pseudomonas fluorescens</u>	heterotrophic	aerobic	+++
<u>Saccharomyces cerevisiae</u>	heterotrophic	facultative anaerobic	++
<u>Aspergillus niger</u>	heterotrophic	aerobic	+++
<u>Leptothrix discophora</u>	facultative autotrophic	aerobic	++
<u>Clostridium tetanomorphum</u>	heterotrophic	anaerobic	+

* As determined by visual optical density:
 + = slight; ++ = moderate; +++ = marked

Table No. 38 - Growth of microorganisms on RM9 medium.

ORGANISMS	ENERGY SOURCE*	O ₂ RELATION**	GROWTH ^φ AT	
			24 HR.	48 HR.
<u>Saccharomyces cerevisiae</u>	H	F	-	+++
<u>Xanthomonas beticola</u>	H	A	+	++
<u>Salmonella choleraesuis</u>	H	F	-	+++
<u>Bacillus subtilis</u>	H	A	-	++
<u>Alcaligenes viscolactis</u>	H	A	-	+++
<u>Rhodotorula glutinis</u>	H	F	-	+
<u>Aerobacter aerogenes</u>	H	F	+++	+++
<u>Pseudomonas maculicola</u>	H	A	-	+
<u>Escherichia coli</u>	H	F	+	+++
<u>Candida albicans</u>	H	F	+	++
<u>Erwinia carotovora</u>	H	A	++	+++
<u>Rhodospirillum rubrum</u>	A H	An A	-	+++
<u>Sarcina lutea</u>	H	A	++	+++
<u>Staphylococcus epidermidis</u>	H	F	-	+
<u>Staphylococcus aureus</u>	H	F	+	+

*

H = heterotroph; A = autotroph

** A = aerobic; An = anaerobic; F = facultative anaerobic

^φ As determined visually: + = slight; ++ = moderate; +++ = marked



VI. INTEGRATED BIODETECTION SYSTEM FOR PHOSPHATE UPTAKE, SULFATE UPTAKE, AND ATP PRODUCTION

A. Integrated Biodetection System

RM9 medium less labeled sulfate ($^{35}\text{SO}_4\text{-S}$) was used for determining ATP production and phosphate, while RM9 plus $^{35}\text{SO}_4\text{-S}$ was used for sulfate uptake studies. As far as constructing feasible models of separate life detection instruments is concerned, this arrangement has been promising. However, there is a possibility that further improvement can be made to simplify and to integrate the biodetection systems so that the instrument may be reduced in weight and perhaps operate more efficiently. With these points in mind, a further improvement on the determination of phosphate and sulfate uptake and ATP production as means for biodetection was attempted.

The improved system utilized the following sequence:

1. Microorganisms from pure culture or soil are inoculated into RM9 medium plus $^{35}\text{SO}_4\text{-S}$ (the same as for sulfate uptake studies).
2. At desired time intervals, an aliquot cell suspension is sampled and filtered through an HA membrane filter.
3. The cells which take up $^{35}\text{SO}_4\text{-S}$ and are retained by the membrane are washed to remove excess $^{35}\text{SO}_4\text{-S}$ and ATP.
4. The cells, plus the HA membrane, are dried and then counted by a radioactive counter for the sulfate uptake measurement.
5. The ATP in the dried cells on the HA membrane from step 4 is extracted by 90% DMSO in Tris buffer, pH 7.4 and the aliquot is injected into the reaction mixture (luciferase, luciferin and MgSO_4) for the ATP determination.



6. The residual phosphate in the filtrate from step 4 is precipitated by ^{14}C -triethylamine-ammonium molybdate-perchloric acid mixture.

7. The ^{14}C -triethylamine-phosphomolybdate precipitate is then washed by non-labeled triethylamine-ammonium molybdate-perchloric acid mixture to remove excess $^{35}\text{SO}_4\text{-S}$ and ^{14}C -triethylamine in the precipitate.

8. The precipitate is then dried and counted by a radioactive counter for phosphate determination.

Three questions arose from attempting to improve the system. These are:

1. What effect will the infrared drying process have on the recovery of ATP from dried cells?

2. How much washing is required to decontaminate $^{35}\text{SO}_4\text{-S}$ on ^{14}C -triethylamine-phosphomolybdate precipitate?

3. Will $^{35}\text{SO}_4\text{-S}$ extracted from cells by DMSO interfere with ATP light response?

To answer the first question, an E. coli culture was selected for the test. Six 1-ml. aliquots of 24-hour cultures of E. coli cell suspension in RM9 plus $^{35}\text{SO}_4\text{-S}$ were filtered through the HA membrane filters in a Swinnex filter unit. Each filter was then washed with 2 ml. 0.05 M Tris buffer. One set of duplicate washed filters was dried under infrared for 10 minutes. The second set was air-dried for 30 minutes and the third set was tested without drying. Each filter was dissolved in 1-ml. of 90% DMSO for ATP



extraction. Ten λ were injected into 100 λ reaction mixture. The average ATP response for each treatment is shown in Table No. 39. The infrared-dried sample had a value (280 mv.) close to the untreated sample (315 mv.). The air-dried sample had the least recovery (240 mv.). Based on this experiment, infrared-dried cells for $^{35}\text{SO}_4$ -S uptake studies apparently can also be used for ATP determination.

To answer the second question - the interference of $^{35}\text{SO}_4$ -S on the determination of phosphate uptake, four one-milliliter aliquots of RM9 medium plus $^{35}\text{SO}_4$ -S (60,000 cpm/ml.) were precipitated with excess non-radioactive triethylamine plus ammonium molybdate-perchloric acid mixture. Each precipitate suspension was filtered through an HA membrane filter and washed with 1, 2, 5, and 10 ml. of triethylamine-ammonium molybdate-perchloric acid washing mixture. The counterparts of non-radioactive sulfate in RM9 were similarly precipitated and the cpm of each precipitate, after being washed, was recorded. The result is shown in Table No. 40. A slight amount of $^{35}\text{SO}_4$ -S apparently was adsorbed by the phosphate-triethylamine complex, despite the washing. Although the $^{35}\text{SO}_4$ -S appeared to interfere slightly with the phosphate determination, the extent was not great enough to render invalid phosphate uptake studies, since the criterion for presence and absence of microorganisms for this test is based on the different degree of phosphate uptake in experimental cultures vs. poisoned cultures. The phosphate uptake in the test cultures generally have been four to eight times higher than in the poisoned control.

Table No. 39 - Recovery of ATP in cells dried
by infrared and by air-drying.

<u>TEMPERATURE</u>	<u>I RESPONSE</u>	<u>RECOV</u>
	mv.	
Infrared-Drying	280	89
Air-Drying	240	76
Control (not dried)	315	100

Table No. 40 - Radioactive count (cpm) of washed phosphate precipitate from RM9 plus $^{35}\text{SO}_4\text{-S}$.

<u>MEDIA</u>	<u>CPM PER ML.</u>	<u>VOLUME (ML.) OF WASHING</u>			
		<u>1</u>	<u>2</u>	<u>5</u>	<u>10</u>
RM9 + $\text{SO}_4\text{-S}$	-	18	18	18	32
RM9 + $^{35}\text{SO}_4\text{-S}$	60,000	199	180	122	126



To reduce the $^{35}\text{SO}_4$ -S interference, the $^{35}\text{SO}_4$ -S in the RM9 has to be decreased, so there will be less chance for undesirable "adsorption" of $^{35}\text{SO}_4$ -S by the phosphate-triethylamine complex. Table No. 41 shows the results of phosphate precipitation studies using 6000, 600 or 60 cpm in RM9 medium. The cpm for the phosphate precipitate was close to background count. By reducing the initial $^{35}\text{SO}_4$ -S radioactivity level, the interference of $^{35}\text{SO}_4$ -S on phosphate determination can be reduced or eliminated.

B. Hazleton Instrument

All the radioactivity counts of sulfate and phosphate uptake studies reported above were made on radioactive counters such as the Nuclear Chicago D-47 and the Widebeta, and the ATP determination was done on the EMR instrument. In order to demonstrate the possibility of integrating the detection of sulfate and phosphate uptake and ATP production, a laboratory instrument, referred to as the Hazleton instrument, was designed. A description and operational procedures are given in Appendix I.

1. ATP Determination

Basically, the ATP determination is not much different from the method described in Section IV, using the EMR instrument. However, the volumes of sample and reaction mixture, calibration of a standard ATP curve and the display of the response had to be investigated.

a. Volumes of Sample and Reaction Mixture

The dimensions of the reaction chamber are 1 inch in diameter and 5/8 inch deep in contrast to 6 mm. diameter and 50 mm. glass cuvette for the EMR instrument. Therefore, it is necessary

Table No. 41 - Radioactive count (cpm) of washed phosphate precipitate from RMD plus different levels of $^{35}\text{SO}_4\text{-S}$.

<u>CPM PER ML.</u>	<u>CPM OF PHOSPHATE PRECIPITATE*</u>
6000	41
600	32
60	16

*

The precipitates were washed with 2 ml. of washing mixture. The background count was 17 cpm.



to establish a volume ratio of ATP sample vs. reaction mixture to obtain the best light response. Standard ATP solution, 10^{-5} $\mu\text{g.}$ per 10λ was prepared in 90% DMSO and 25, 50, 75, or 100λ volume were injected into the corresponding 10 times volume of reaction mixture. The results are shown in Table No. 42. The highest response was obtained from injecting 10λ ATP standard into 100λ reaction mixture, consequently this ratio was selected for all ATP tests using the Hazleton instrument. For this experiment the photomultiplier had glass window only. No scintillator crystal was used. The response was displayed on a strip chart recorder. (Brush Model 10).

b. Standard ATP Curve

An ATP standard curve was prepared using 100λ ATP solution. and 1000λ reaction mixture.

Standard ATP solution was made from 1 mg. per ml. stock solution:

- I. 0.1 ml. stock solution + 9.9 ml. 90% DMSO = 1 $\mu\text{g.}$ per 100λ
- II. 0.1 ml. of I solution + 9.9 ml. 90% DMSO = 10^{-2} $\mu\text{g.}$ per 100λ
- III. 0.5 ml. of II solution + 4.5 ml. 90% DMSO = 10^{-3} $\mu\text{g.}$ per 100λ
- IV. 0.5 ml. of III solution + 4.5 ml. 90% DMSO = 10^{-4} $\mu\text{g.}$ per 100λ
- V. 0.5 ml. of IV solution + 4.5 ml. 90% DMSO = 10^{-5} $\mu\text{g.}$ per 100λ

The standard curve is shown in Figure No. 19 for ATP concentrations ranging from 10^{-5} $\mu\text{g.}$ per 100λ up to 10^{-2} $\mu\text{g.}$ per 100λ . The responses are expressed in mv.

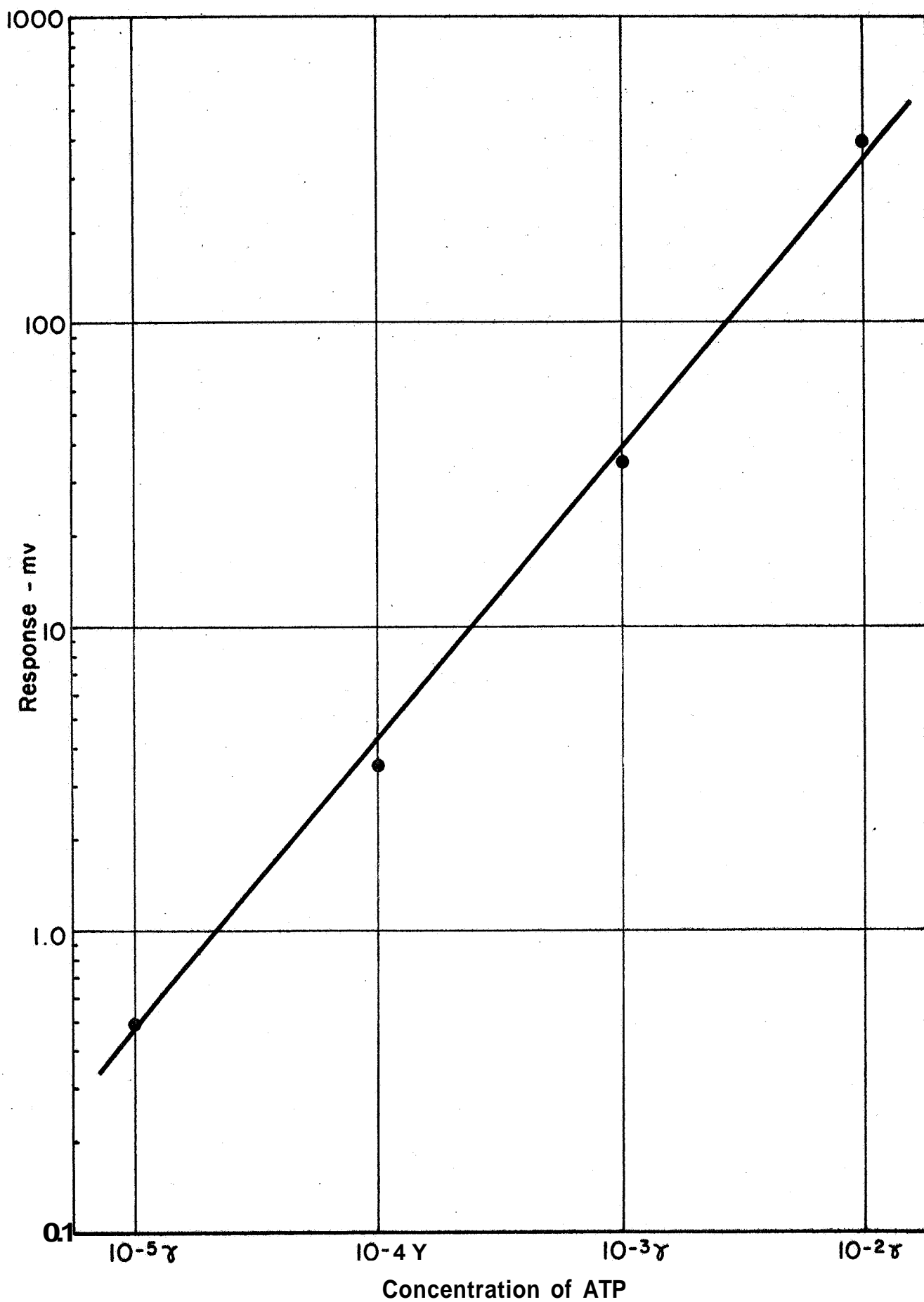


Figure No.19 - Standard ATP curve on Hazleton Detector

Table No. 42 - Volumes of ATP sample and reaction mixture for ATP response using Hazleton instrument ■

<u>ATP IN 90% DMSO</u>	<u>REACTION MIXTURE</u> <u>μl</u>	<u>NET RESPONSE</u> <u>mv.</u>
10^{-3} g/100 <i>μl</i>	1000	45
7.5×10^{-4} μg/75 <i>μl</i>	750	28
5.0×10^{-4} μg/50 <i>μl</i>	500	13
2.5×10^{-4} μg/25 <i>μl</i>	250	7



2. Radioactivity Count

A comparison of the radioactivity counting between the Nuclear Chicago D-47 Gas Flow and the Hazleton Instrument is given in Tables No. 43 and No. 44. Three scintillation crystals to detect the weak β -radiation have been tested in the Hazleton Instrument. These are .25" $\text{CaF}_2(\text{Eu})$ with .005" Al window, .25" $\text{CaF}_2(\text{Eu})$ without window and .015" anthracenes. The tests with the Hazleton Instrument showed that the anthracene scintillator was the best of all, having a low background count. The $\text{CaF}_2(\text{Eu})$ crystals should equal, or exceed, the performance of the anthracene if the thickness is optimized for ^{14}C and ^{35}S beta and the time constant of the associated electronics increased to match the crystal. In the following studies, all the radioactivity counting using the Hazleton Instrument was with the anthracene crystal.

C. ^{35}S Interference on ATP

In regard to the third question asked in Section VI, A, above; since the photomultiplier-scintillator system used for detecting ATP and phosphate is also sensitive to β -radiation of $^{35}\text{SO}_4\text{-S}$, the amount of $^{35}\text{SO}_4\text{-S}$ radiation may become additive to ATP light response. If this is the case, the background noise for the ATP light response will be increased. The following experiment was conducted to evaluate the extent of $^{35}\text{SO}_4\text{-S}$ effect on ATP response.

The stock $^{35}\text{SO}_4\text{-S}$ solution, having radioactivity of 19850 cpm per ml., was diluted 10 times with 100% DMSO, to make approximately 2000 cpm per ml. in 90% DMSO. One-tenth ml. of this labeled sulfate

Table No. 43 - Phosphate, sulfate uptake and ATP production of E. coli culture in RM9-35SO₄ medium

[illegible]

* All the counts are corrected for background count.

A: Counted by Hazleton Instrument

B: Counted by the Nuclear Chicago D-47 Gas Flow Counter

* NR = No response

Table No. 44 - Phosphate, sulfate uptake and
ATP production of culture from
soil sample in RM9 $^{35}\text{SO}_4$ medium

SYSTEMS	CELL DENSITY No. per ml.	PO ₄ -P*		³⁵ SO ₄ -S		** ATP mv.	CELL DENSITY No. per ml.	PO ₄ -P		³⁵ SO ₄ -S		CELL DENSITY No. per ml.	PO ₄ -P		³⁵ SO ₄ -S		ATP mv.	
		A	B	A	B			A	B	A	B		A	B	A	B		
Medium Control	0	839	1026	62	81	NR	0	938	1335	58	63	NR	0	592	896	45	64	NR
Soil Culture	5 x 10 ³	2209	2808	87	102	2.5	2.8 x 10 ⁸	793	1548	560	720	3	5.1 x 10 ⁸	338	577	1640	2091	4
Poison Control (0.01% HgCl ₂)	5 x 10 ³	2313	2542	83	107	NR	0	1015	1322	167	189	NR	0	1573	2632	100	111	NR
Poison Control (1.5% perchloric acid)	5 x 10 ³	2253	2425	79	86	NR	0	627	870	55	52	NR	0	786	1077	125	185	NR
Standard ATP 10 ⁻⁴ per 100A							4						1					1

* All the counts are corrected for background count

A: Counted by the Hazleton Instrument

B: Counted by the Nuclear Chicago D-47 Gas Flow Counter

** NR = No response



solution was injected into 1 ml. of reaction mixture in the Hazleton Instrument for light response. The detector for the ATP reaction also had an anthracene crystal for detecting the weak β -radiation. No light response was detected in this experiment, the reason being, probably, that the β -radiation given off by $^{35}\text{SO}_4\text{-S}$ was absorbed by the liquids (reaction mixture and DMSO solutions), thus no radiation could reach the scintillator.

D. Residual ATP and $^{35}\text{SO}_4\text{-S}$

Because of the limitation in weight for a life detection instrument for a space mission and the nature of time sequence required for phosphate, sulfate and ATP metabolism analyses, reaction chambers or a pipeline of the instrument may have to be reused several times. The following experiments were made to show whether contamination will be a problem, and if so, to develop an adequate washing process.

1. Residual ATP in Reaction Chambers

A standard ATP solution 10^{-2} $\mu\text{g. per } 100\lambda$ in 90% DMSO was injected into 1000λ reaction mixture in the reaction chamber of the Hazleton Instrument. After the reaction was over, 100λ of the spent ATP reaction mixture was injected into another clean reaction chamber containing fresh reaction mixture to see if there was residual ATP from the previous experiment. No response was obtained, indicating that practically all the ATP had been converted to ADP. If any ATP had been left, the concentration was less than 10^{-5} $\mu\text{g. per } 100\lambda$, since this is the sensitivity of the Hazleton Instrument. (Figure No. 19). Based on this experiment, rinsing with water should be adequate to remove



trace amounts of ATP, if any, from a used reaction chamber.

2. Residual $^{35}\text{SO}_4\text{-S}$

One ml. of E. coli culture in RM9- $^{35}\text{SO}_4$ medium was filtered through HA membrane in a Swinnex filtration unit. After removal of the filtration unit, the same syringe was used to draw up 1 ml. of Tris buffer for washing the residual cells left in the syringe,. A new filtration unit containing a new clean HA filter was attached to the same syringe and the Tris buffer plus cells were filtered through the HA membrane. This constituted the first washing. The filter plus cells was counted by a radioactive counter. The second washing was similarly done and the HA plus cells, if any, were counted again. The washing was done a total of eight times. The original culture plus filter had a radioactivity of 3852 cpm, while the first, second, third, and fourth washings had respectively, 12, 13, 12 and 4 cpm. No radioactivity was detected in the fifth washing through the eighth. Although the residual cells in the syringe were negligible, (since the first washing only showed 12 cpm) four washings of 1 ml. volume each were required to remove the radioactivity entirely.

E. Phosphate Standard Curves From RM9- $^{35}\text{SO}_4$ Medium

A standard curve of phosphate ions prepared by the ^{14}C -triethylamine method has been described in Section 11, A, 2, and is shown in Figure No. 4. The radioactivity of 30 cpm and 140 cpm for ^{14}C -triethylamine-phosphomolybdate precipitate, represent, respectively, 100 $\mu\text{g.}$ and 1000 $\mu\text{g.}$ phosphate-P per liter. The precipitate was prepared by adding 0.05 ml. of ^{14}C -triethylamine solution (specific activity of 1.1 mc.



per μM in 0.064 ml. was diluted 1:100 with distilled water and then 1:10 with 0.8 M unlabeled triethylamine), 0.05 ml. of 4 N perchloric acid and 0.25 ml. 0.08 M ammonium molybdate into 1 ml. of various phosphate concentrations in RM9 medium. The radioactivity was rather low (25 cpm) for 100 μg . phosphate-P per liter. Since it is desirable to have higher radioactivity in the precipitate, preparation of such precipitates was attempted.

Figure No. 5 showed that higher cpm of the precipitate was obtained by increasing the amount of ^{14}C -triethylamine solution in the precipitation process. One-hundred and seventeen mg. of the ^{14}C -triethylamine, with specific activity 0.59 mc. per μM , was dissolved in 0.63 ml. of distilled water and then subsequently diluted 1:50 with 0.8 M of unlabeled triethylamine. The highest cpm from phosphate in 1 ml. RM9 - $^{35}\text{SO}_4$ occurred when using 0.5 ml. of ^{14}C -triethylamine solution. Further increase of ^{14}C -triethylamine did not increase the radioactivity of the precipitate. Based on this experiment it was decided that 0.2 ml. and 0.5 ml. of ^{14}C -triethylamine should be tried for making phosphate standard curves of higher radioactivity. Two-tenths or 0.5 ml. of ^{14}C -triethylamine, along with other ingredients as described above, were added to 1 ml. of various phosphate concentrations in RM9 - $^{35}\text{SO}_4$ medium. The standard curves are shown in Figure No. 20. Increase in the amount of ^{14}C -triethylamine did increase the radioactivity of the precipitate and the curves appear to be parallel.

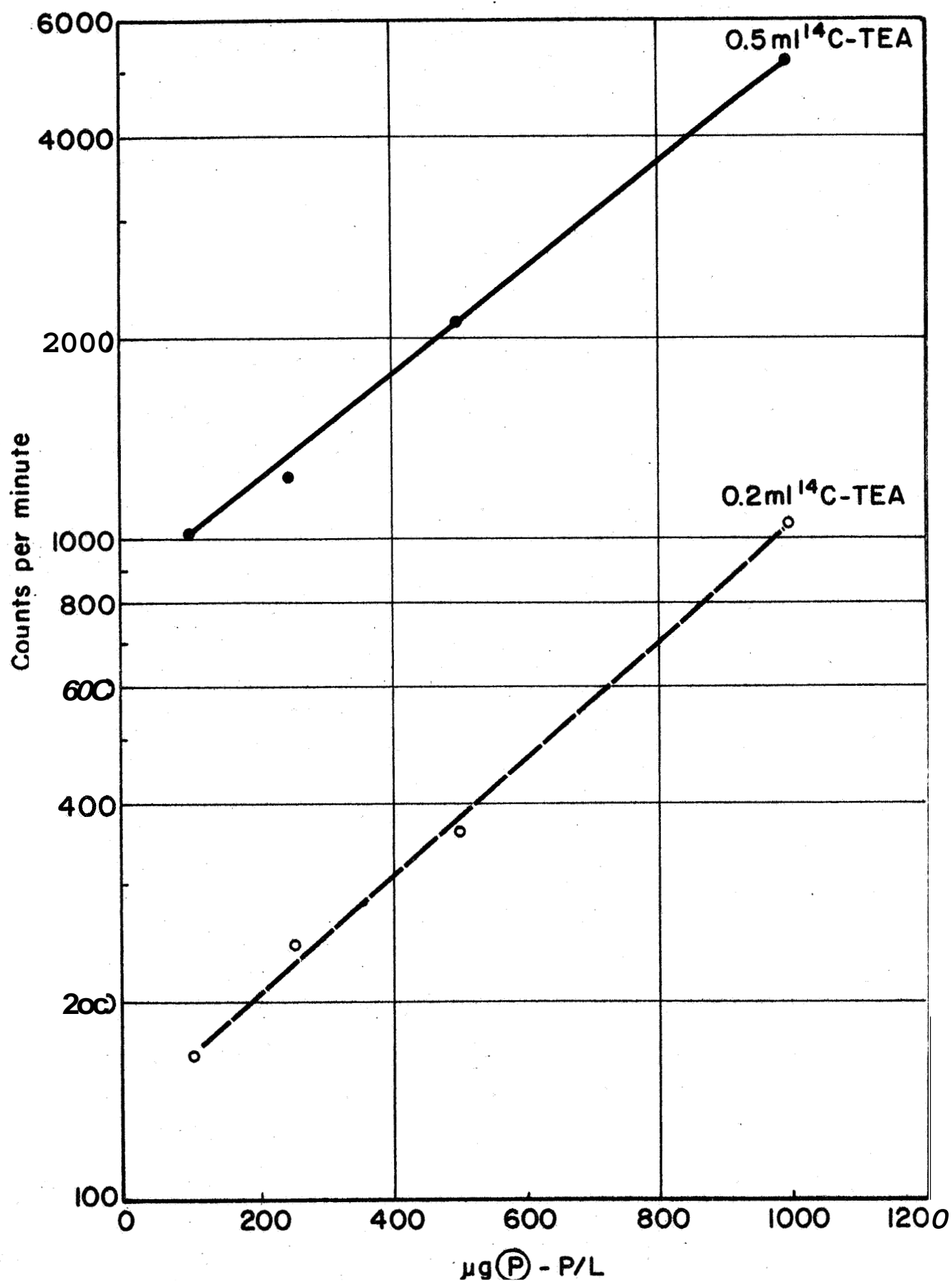


Figure No.20- Standard curves of Phosphate - P in ml of RM9 - $^{35}\text{SO}_4$ using different volumes of ^{14}C - Triethylamine



F. Pure Culture Study Using the Hazleton Instrument

An E. coli culture was selected for studying the integrated scheme for the determination of microbial activity. The medium used was composed of 39.5 ml. of RM9, and 0.5 ml. of stock $^{35}\text{SO}_4$ solution. A RM9- $^{35}\text{SO}_4$ medium, poisoned (HgCl_2) control and a cold (non-radioactive) medium of RM9 (less $^{35}\text{SO}_4$ -S) were also included for comparison. One-tenth ml. of E. coli washed culture, containing 2.4×10^6 cells, was added to 40 ml. of each of the above three media and incubated statically at 26°C . A medium control was also included for the analyses. At 0, 3, 24, and 48 hours, one-ml. aliquots in duplicate, were filtered through a Swinnex filter using a 13 mm. diameter HA membrane. The cells plus membrane were washed with 2 ml. of saline and then dried for 10 minutes under an infrared lamp. The sulfate uptake was counted by both the D-47 gas flow counter and the Hazleton Instrument. After the radioactivity count, the dried cells plus membrane were extracted with 1 ml. of 90% DMSO for the ATP determination. One hundred λ was injected into 1000 λ of the reaction mixture.

The filtrate (1 ml. from the filtration of the cell culture) was mixed with 0.05 ml. of 4 N perchloric acid, 0.25 ml. of 0.08 M ammonium molybdate and 0.25 ml. of ^{14}C -triethylamine for phosphate determination. Radioactivity counts by both instruments were included. The results are shown in Table No. 43. Although there were some discrepancies in the radioactivity as counted by the Hazleton Instrument and by the Nuclear Chicago D-47, the trend was comparable. At 24 hours, phosphate uptake in both of the E. coli cultures was evident,



compared with the poisoned control. The $^{35}\text{SO}_4$ uptake was rather pronounced in the E. coli culture with RM9- $^{35}\text{SO}_4$ medium. No sulfate uptake was shown in the poisoned culture. The ATP response was definitely shown in both of the E. coli cultures, but not in the poisoned culture or medium control. The evidence of microbial growth was positively demonstrated in this experiment.

G. Soil Culture Study Using Hazleton Instrument

Ten grams of moist loam soil were placed in 50 ml. of sterile saline. After stirring for 10 minutes, the suspension was settled for 5 minutes and 0.5 ml. of the supernatant was pipetted and inoculated into 40 ml. RM9- $^{35}\text{SO}_4$ medium. Poisoned controls of 0.01% HgCl_2 and of perchloric acid (0.6 ml. per 40 ml. medium) were also included. Incubation was made statically at 26°C . At 0, 24, and 96 hour incubation, 1 ml. aliquots were filtered for sulfate and phosphate uptake and for ATP production analyses as described in the previous section. The microbial population was estimated by the spread plate technique using Tryptic Soy Agar medium. The results are shown in Table No. 44. The loam soil must contain a rather high phosphate ion concentration, because at 0 time, the phosphate concentrations in all the cultures inoculated with soil samples were twice as high as the medium control. Nevertheless, phosphate uptake was evident in the soil culture samples after 96 hours incubation. The poisoned culture at 48 hours contained at least three to five times as much phosphate as the soil culture, indicating phosphate uptake by the latter. The sulfate uptake and ATP production were very distinctly shown in the



soil cultures after **24** and 96 hours incubation.

VII. ENGINEERING CONCEPT

A. A Description of the Biochemical Process to be Mechanized

The biochemical research was aimed at determining the feasibility of measuring phosphate uptake, sulfate uptake, and ATP content of any organisms which might be collected from the Martian surface and cultured in the surface laboratory. Also, it was hoped to learn if these values, measured after specific time intervals from beginning the culture, could be used to establish profiles of metabolic activity, thereby providing strong evidence that microorganisms had been discovered and grown.

These objectives were found to be biochemically feasible, at least for terrestrial organisms.

The biochemical processes which would need to be mechanized if such an instrument were built, are shown in the block diagram in Figure No. 21. A considerable effort has been made, including laboratory tests (Sections II through VI above), to integrate and simplify these processes. The conceptual design of the Automated Microbial Metabolism Laboratory (AMML) presented here will be based on the processes illustrated in Figure No. 21. It is possible that further simplification can be achieved and thus make it desirable to modify the conceptual design at some time in the future.

A soil sample is collected and an inoculum prepared by filtering and mixing with distilled water. The resulting suspension is then introduced into the incubation chambers. Since the biochemical

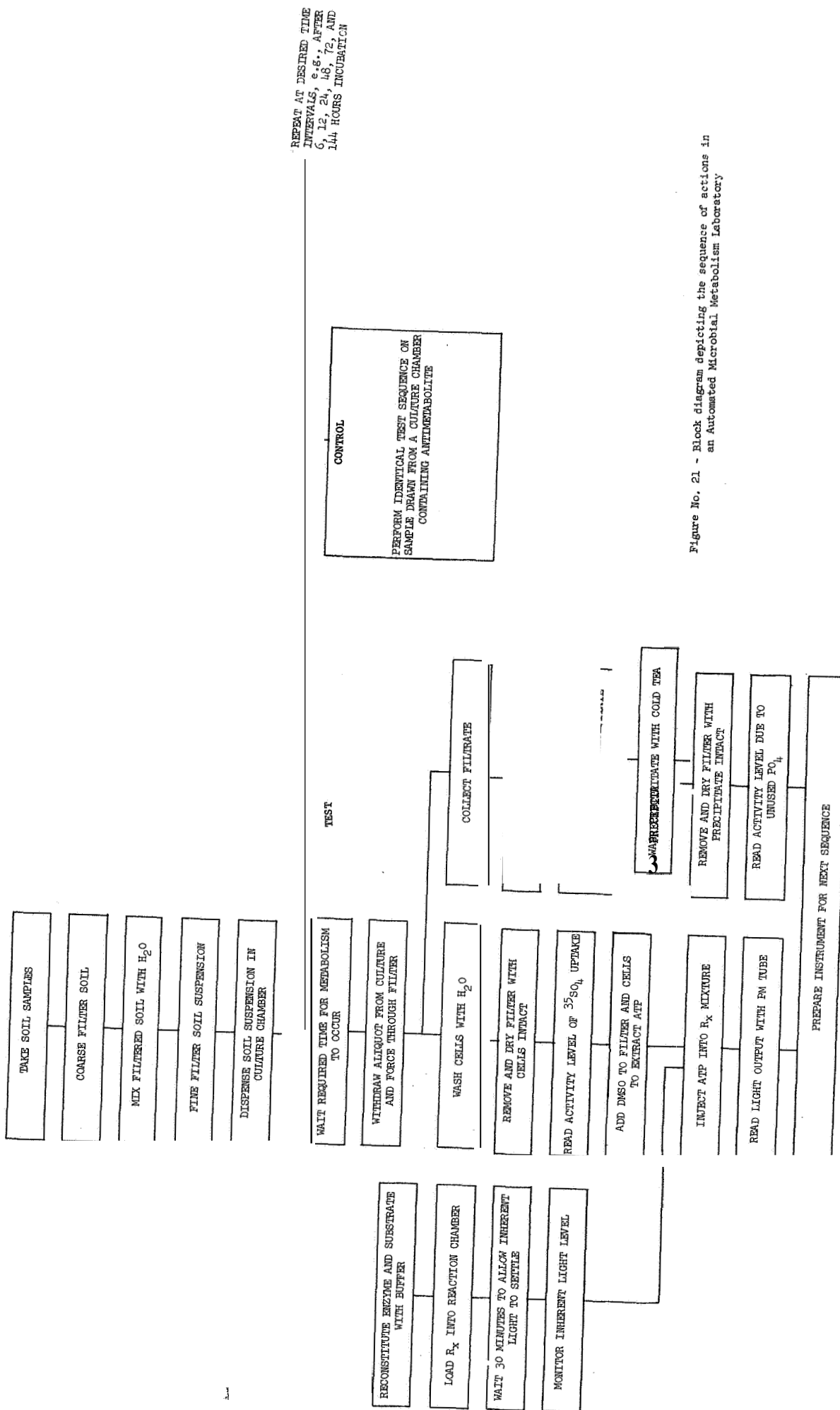


Figure No. 21 - Block diagram depicting the sequence of actions in an Automated Microbial Metabolism Laboratory



tests have shown it possible to use a single growth medium for both the sulfate uptake and phosphate uptake experiments and further, that cells dried for measuring ^{35}S content can subsequently be used in assaying for ATP, only two culturing chambers are required. One of these chambers contains the growth medium and inoculum, and the other is identical except that an antimetabolite (poison) has been added. The second chamber is used as an experimental control, indicative of what occurs when no metabolism is likely.

The test sequence is identical for both samples, test and control. An aliquot is removed and filtered to remove the cells. Any organisms removed are first washed, then dried on the filter and read directly with a nuclear particle detector for ^{35}S taken up from the medium. The filtrate is retained and radioactive triethylamine (TEA), ammonium molybdate, and perchloric acid added to precipitate the unused phosphate. The TEA is labelled with ^{14}C and supplied in excess. Therefore, the precipitated, unused phosphate will be tagged proportionately to the amount. This precipitate is then washed with untagged TEA, dried and counted.

The ATP experiment is performed on the cells removed for analysis of sulfate uptake. Dimethylsulfoxide (DMSO) is added to extract ATP, then the ATP is injected into an excess R_x mixture (reconstituted enzyme, substrate, and MgSO_4 in Tris buffer). The inherent light of the R_x and the gross light output after the injection of ATP are both read with a photomultiplier (electron multiplier phototube).



It is also possible to integrate equipment into this system which could be used to conduct Gulliver-type experiments (Ref. 1), as well as experiments designed to look for evidence of photosynthetic activity. If this were done, another growth medium and culture chamber would need to be added since Gulliver offers a substrate labelled with ^{14}C to the microorganisms collected and then monitors the output of $^{14}\text{CO}_2$. If a light source is added to the culture chamber, evidence of the presence of both heterotrophic and autotrophic organisms can be obtained by measuring increasing CO_2 output with the light off and leveling off or decreasing CO_2 output with the light on. If this experiment were added to the AMML and conducted in the spacecraft, some redesigning of the Gulliver equipment may be desirable, since it may be possible to use the same nuclear particle detector to read SO_4 uptake, PO_4 uptake, and CO_2 output, or respiration, as well as the same spacecraft sample acquisition system. If the experiment (Gulliver and photosynthesis) were conducted in situ, (Ref: 1), then the only modification needed would be the addition of a light source and possibly the redesigning of the detector and associated chemical getter to allow observations over longer periods of time.

B. General Operational Requirements

In addition to requirements and limitations established by the biochemical processes involved in an Automated Microbial metabolism laboratory, the extraterrestrial environments which it must transit and in which it must operate, present stringent restrictive conditions greatly affect the engineering design of the device.



While definitive specification of many such restrictions are necessarily dependent on the spacecraft system design and mission details, their general consideration bears directly on the AML engineering problem to be investigated.

1. Launch Acceleration

Acceleration at launch, both vibrational and steady state, should impose no greater requirement on this system than on any other electromechanical system. Mechanical and electrical components should be selected to meet the usual acceleration criteria for space application. In the system's mechanical design, particular consideration must be given to the selection of valves and to the tubing and piping layout to preclude acceleration-induced failure.

2. Flight Time

The flight time for a trip to Mars, approximately eight months, imposes a severe requirement on the biochemical elements of the system. Four areas are affected:

- a. Radioactive tracers with half-life short compared to eight months, cannot be used, since their activity would be too low on arrival.
- b. Special precautions must be taken to maintain the stability of the reagents and especially to minimize degradation of enzymatic activity during the flight.
- c. Particular care must be exercised in the initial sterilization since the system will contain metabolic nutrients which could conceivably nurture any microorganisms inadvertently introduced prior to launch.
- d. Consideration must be given to long term effects of what are normally rather inactive chemicals, especially the corrosive



effects of reagents during this storage.

3. Temperature

Again, it is the biochemical portion of the system which suffers most significantly from extreme temperature excursions. The undesirable effects may include inactivation of enzymes, degradation of stability, acceleration of corrosive or other unwanted activity and irreversible changes in both physical and chemical properties. Generally, excessively high temperatures would appear to be most detrimental, but this is not to rule out the possibility that prolonged exposure to very low temperatures may have adverse results.

a. Pre-flight

Prior to insertion in the spacecraft, critical chemical reagents can be kept at any desired temperature and thus protected from deleterious temperature effects. However, immediately prior to launch, the spacecraft will be subjected to a sterilization cycle employing dry heat for approximately 24 hours. This exposure would unquestionably destroy the enzymatic activity; therefore, an acceptable substitute sterilization procedure for the enzyme must be provided.

The location of the AMML within the spacecraft must take into consideration the problem of post-sterilization insertion of temperature-sensitive reactants. These reactants must be protected, during countdown and launch, from overheating due to absorption of heat from the spacecraft and its electronics, from the ambient, or from any other source.



b. In Transit

Assuming the spacecraft will be given a specific orientation during flight, as was Mariner IV, for example, the location of AML within the craft must also take into consideration the direction of the sun. Ideally, radiant panels associated with containers of temperature-sensitive reactants should "look at" cold space during the long flight to Mars.

c. On Mars

The specific diurnal temperature range will depend upon the site of the landing. Near perihelion, noon tropical temperatures may be expected to have a maximum of around 32° C. Consequently, the enzyme may be protected against overheating with no more than reasonable insulation. On the other hand, minimum night-time temperatures of as low as -100° C. might be reached with resultant freeze-up of fluid systems unless the AML is warmed.

4. Gravity On The Martian Surface

Gravity on the Martian surface is about 392 cm/sec^2 , that is, about 40% of Earth's. Any gravity-dependent operations (e.g., fluid flow) must consider the lower gravity of Mars.

5. Radiation

a. In transit, the spacecraft will pass through the radiation belts associated with the Earth. However, no comparable exposure is anticipated in the vicinity of Mars. During the whole trip, the craft is subject to galactic cosmic radiation and to solar radiation.



b. On the surface of Mars, the craft will continue to be subject to the primary cosmic radiation. The contents may be subjected to secondary cosmic radiation. Such radiation would appear as background for the sulfate and phosphate uptake procedures.

6, Martian Terrain

The terrain upon which the spacecraft lands could prove to be the determining factor in the success of the detection of life by AMML. Assuming that detectable life does exist on Mars, the presence of live organisms in the particular soil being tested could be chancy, especially if life is sparse. Furthermore, the nature of the terrain at the landing site could make sampling difficult or perhaps impossible due either to the constitution of the material at that point or to the detailed topography (for example, a near-vertical slope). An additional factor influencing life detection procedures is the degree of disruption of the surface by the landing. Ideally, the sample should come from an area undisturbed mechanically or thermally by the spacecraft.

7. Attitude of the Spacecraft

It is unlikely that the landing site will be perfectly horizontal. Some tilt can be tolerated by the AMML, but at a cost in weight and complexity which could be prohibitive if gross misorientation is to be compensated. The AMML involves fluid and mechanical elements upon which gravity will have some effect; the degree of allowable tilt will present a design requirement. Ideally, the spacecraft should be self-righting within specified limits-which, for the AMML, need not be precise. For example, the concept for the



AMML presented herein, could probably function at, say, 35° out of the vertical, but not at, say, 135°;

8. Operating Time

There are three distinct operating conditions for the AMML:

- a. For approximately eight months "flight time" the AMML must be in a standby condition, not detrimental to its components, and from which it can be activated at the destination.
- b. For approximately six days at the destination, the AMML must be in an incubation condition, during which temperatures are maintained to promote the growth of any microorganisms and to prevent either freeze-up of the fluids, or degradation of reactants.
- c. During approximately seven assay periods of approximately one-half hour duration each, the AMML will be in an active condition which will require the necessary additional power to transport filters, dry ^{precipitates} ~~filtrates~~, operate valving, read-out assay results and transmit results to the spacecraft telemetry system for retransmission.

C. Discussion of Engineering Problems

An equipment block diagram of an AMML is shown in Figure NO.22. This diagram is not intended to depict a proposed design, but rather the functions which must be performed to mechanize the processes described in Section VII., A. This diagram parallels that of Figure No. 21, but also shows the storage and preparation of reagents, pumping and valving action, the flow of solid and fluid matter, the flow of energy and information as to where filters, driers,

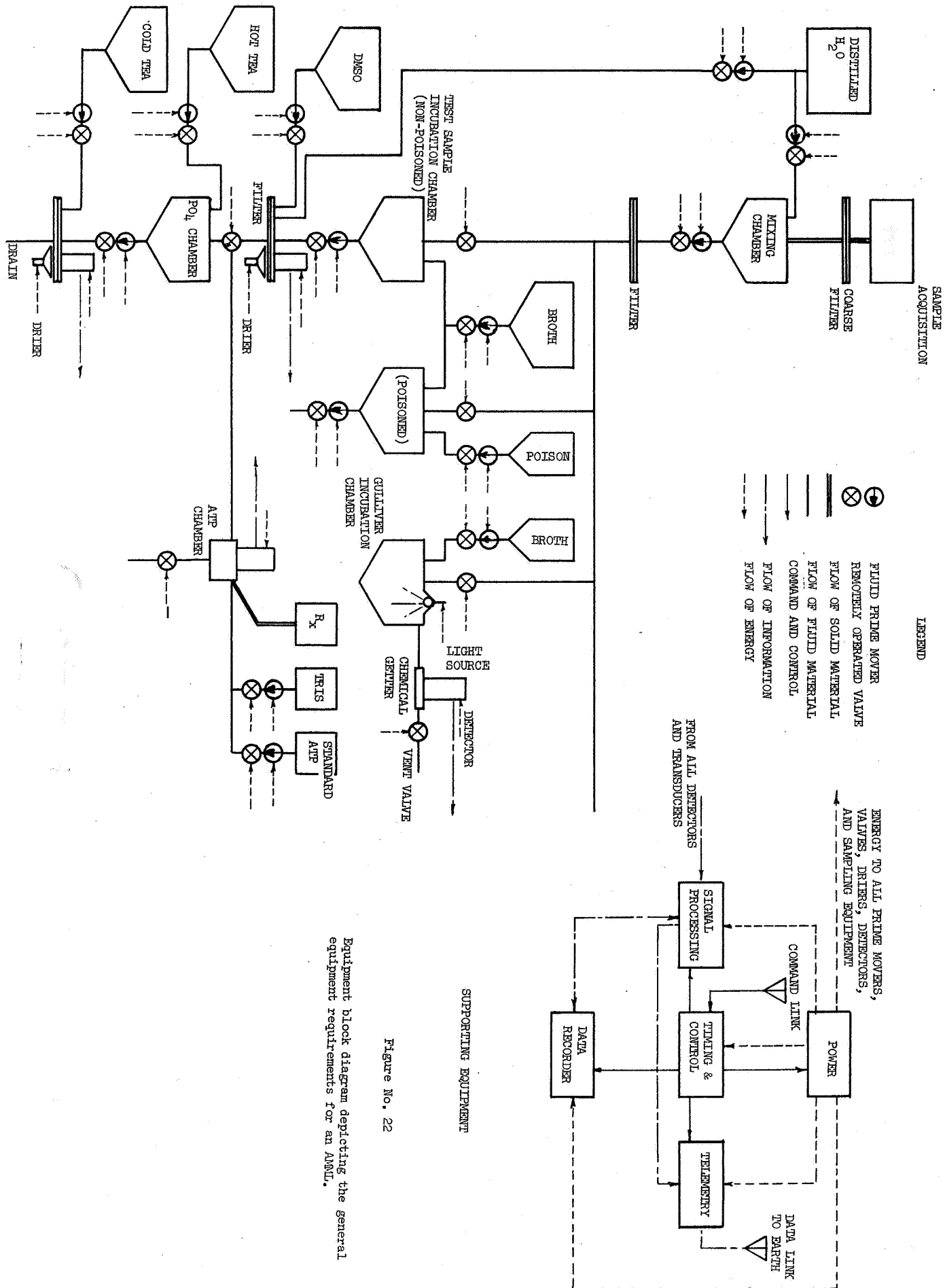


Figure No. 22

Equipment block diagram depicting the general equipment requirements for an AVL.



and detectors are required, and the accumulation and mixing of fluids. Figure No. 22 should be referred to in the following exploration of engineering problems to be encountered in designing an AMML.

1. Storage of Reagents

All the reagents required for use in an AMML must be stored within the spacecraft during the eight/ninth journey to Mars and then dispensed as needed after landing. This can cause difficulty with some of the reagents since they must remain stable throughout this period and they must not react with the material of the storage chambers. If any of the reagents require use at a specified temperature, then equipment must be provided to maintain and control reagent temperatures. The reagents listed in Table No, 45 are required by the proposed AMML and provisions must be made for their storage.

2. Compatibility of Materials

Since there is also a requirement that reagents not react with the materials in which they are stored or transported, additional study and tests of the compatibility of materials must be conducted before an AMML can be completely designed. First, experience at this laboratory has shown that certain metals are toxic to the enzyme. Brass and aluminum both cause inhibition of enzyme system activity. Aluminum oxide, (as the surface of anodized aluminum) is also toxic, although much less so than aluminum. Aluminum and brass should, therefore, be avoided in the design of storage units, piping, fittings,

Table No. 45 - Reagents required by AMML

EACHEN	VOLUMES PER CYCLE		NO. OF CYCLES	TOTAL VOLUME (PLUS PIPING LOSS) ml.	REMARKS
Distilled Water	2.0 ml.		4 x 7 x 2 = 56	124.0	for washing and flushing instrument
Poisson (Bard-Parr)	0.6 ml.		1	0.6	antimetabolite for experimental control mixed in RM9
Dimethyl Sulfoxide, 90%	1.0 ml.		7 x 2 = 14	16.0	for ATP extraction
Tris Buffer, 0.05N, pH 7.4	1.0 ml.		7 x 2 = 14	16.0	for reconstituting enzyme R _x
Luciferase) reconstituted Luciferin) in Tris MgSO ₄)	3.5 mg) 0.2 mg.) 0.82 mg.)	per 1 ml. Tris	7 x 2 = 14		stored mixed and frozen together in one observation measures
Triethylamine, 0.01 M + Perchloric Acid, 0.2N	0.5 ml		1 x 2 = 14	8.0	mixed and stored together for washing PO ₄ precipitate
Triethylamine, ¹⁴ C labeled Perchloric Acid, 4N	0.2 ml. 0.05 ml.		7 x 2 = 14	4.0 1.0	mixed and stored together for precipitating PO ₄
Ammonium Molybdate, 0.08 M	1.0 ml.	(0.5 ml. precipitation and 0.5 ml. washing)	7 x 2 = 14	16.0	for precipitation and washing PO ₄ precipitate
RM9- ³⁵ SO ₄ Medium	2.0 ml.		7 x 2 = 14	40.0	medium for culture



valves, and reaction chambers for the ATP experiment. Anodized aluminum could be used to construct reaction chambers, but when this use is not advisable since it will cause an inhibition of ATP response. Most stainless steels have been found to be very compatible with the reagents of the firefly bioluminescent reaction, although it is not known over what long periods of time this is true. Stainless steel is probably suitable for use in piping, valves, fittings, and reaction chambers for the ATP experiment. It is not known if it is suitable for long term reagent storage. Glass and plastic containers have been used successfully in this laboratory for long term storage of the lyophilized, frozen enzyme. The plastic containers used have generally been some form of polystyrene. The inertness and wide allowable temperature range of teflon (Ref:13) make it appear very promising as a material for long term packaging.

3. Preparation of Reagents

Enzyme, lyophilized, and stored frozen, must undergo some preparation before it can be used in an ATP assay. In the laboratory it has been found that it can be prepared simply by weighing out the correct amount of luciferase and luciferin and reconstituting it with distilled water or Tris buffer. Tris buffer is preferred, since it tends to control the pH of the reagent mixture, as well as diluting it. No mixing is required as long as the mixture can stand 15 minutes. It is already required that the enzyme mixture stand for about 30 minutes to reduce the inherent light output and, thus, the mixing time imposes no new requirement.



Tris buffer can be stored for short time mixed, or indefinitely, dry. Less chance of decomposition or contamination occurs if it is stored dry, but the instrument design can be simpler, if it is stored mixed. By proper sterilization and sealing of containers, it can probably be stored mixed.

4. Transfer of Fluids

In any instrument designed to conduct wet chemical assays such as this one, the method of moving fluids about becomes a very important consideration. Fluid flow is always attendant with pressure differentials which either cause or characterize the flow. Pressure differentials can be created gravitationally, mechanically, or by use of a pressurized gas, including atmospheric pressure.

Pumping systems of the first two types can be used in an **AMML**, but not of the latter type, unaided, owing to the very thin atmosphere on Mars (see Section VII., B.). Actually, gas pressure can be used to move fluid on Mars if the differential is created by raising the pressure at some point above the ambient level.

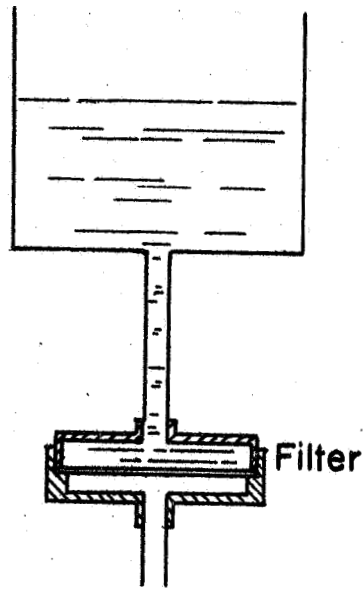
The fluids to be transferred must be metered accurately in very small quantities (0.1 to 2 ml.). This requirement eliminates consideration of any continuous-flow pumping system for fluid transfer and leaves only a few methods as worthy of consideration.

Pipetting may be difficult or impossible because of the problem of very low atmospheric pressure previously mentioned, while syringing appears feasible, provided the reagents ~~to~~ be drawn into



syringes are stored under pressure, and offers the advantage of good metering and positive expulsion. Gravitational flow can be considered only if the spacecraft is capable of erecting itself to within a very small angular displacement of the local vertical (see Figure No. 23). Furthermore, unaided gravitational flow would produce very low flow rates through membrane filters (See Section VII.C.5), especially on Mars. Therefore, it was decided to eliminate fluid transfer by means of an unaided system such as that shown in Figure No. 23. Fluid flow through a filter or other restrictive device can be augmented by adding a pressure source as shown in Figure No. 24. The design is better, however, if the pressure required to transfer the liquid is transmitted through a piston or a bladder. In this case, the liquid is confined allowing operation in any attitude and more precise mensuration. The pressure on the piston could be supplied by a mechanical spring, thus making a pressurized gas supply unnecessary. The advantage in doing this may be offset by the danger of a reactant leak developing during the flight, by the tendency of mechanical springs to permanently deform under sustained tension, and by the possibility that the springs under tension, and subject to low temperatures for eight months may fail.

The pressures which any pumping system must deliver will be determined by the estimated pressure drops against which the fluid must work, with appropriate allowances made for surface tension. Whatever the pressure requirements may be, the required spring constant or the



**Figure No.23 - Simple gravitational flow
filtration system**

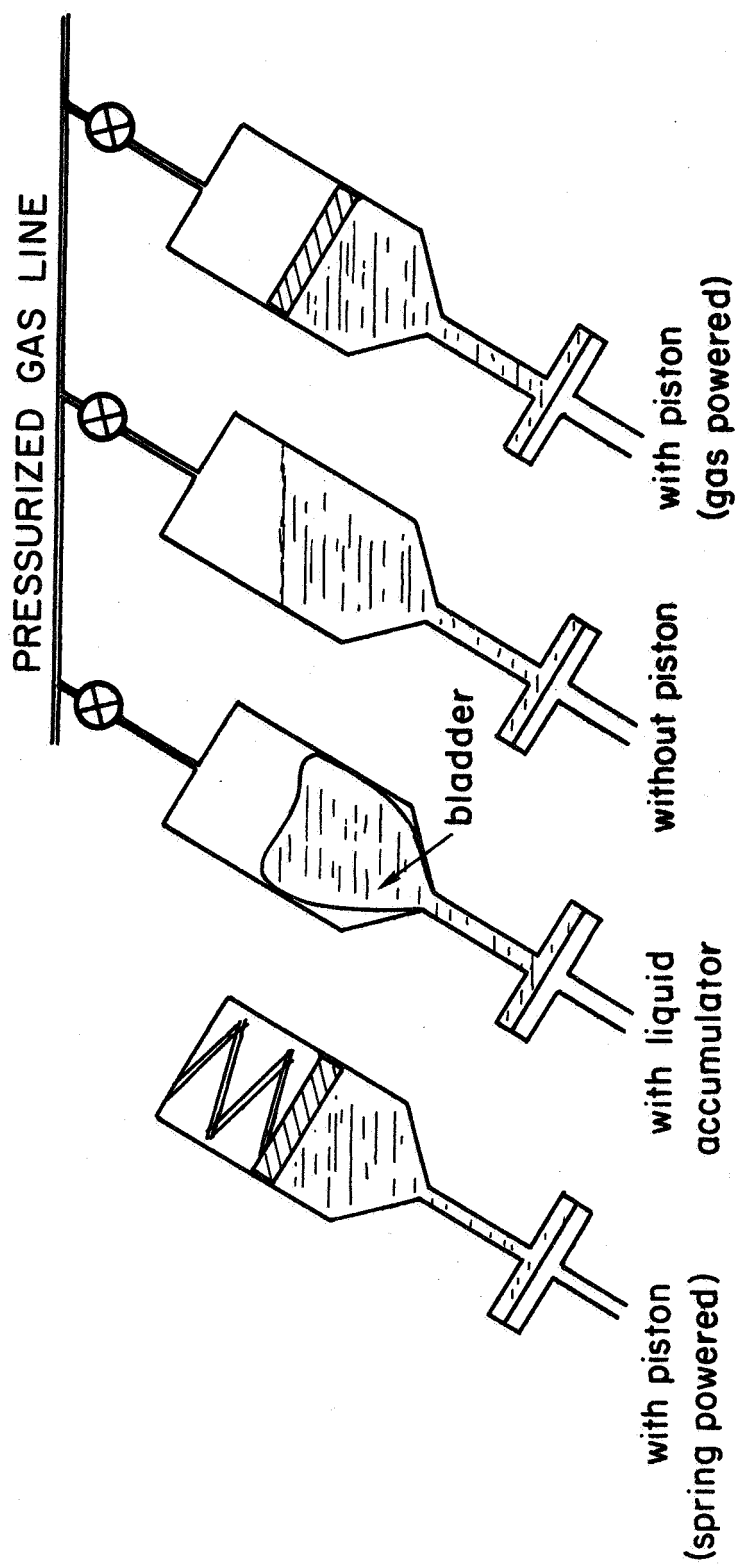


Figure No.24 - Filtration system with flow augmentation



initial pressure of the compressed gas can be readily determined.

Since the spring will be compressed to its maximum displacement when the cylinder is full, the system will deliver its highest pressure at the instant the valve opens. This pressure must be below that value of pressure differential at which the filters are likely to rupture. This system will deliver its lowest pressure when the cylinder reaches its maximum delivered volume. This value will be determined by the minimum desired flow rate for the filter used.

5. Filtration of Cells and Particles

It can be seen from the equipment block diagram (Figure No. 22) that filtration of cells and particulate matter must be performed several times during the course of one operational sequence of the instrument. Filtration was chosen rather than centrifugation in order to simplify the system design as much as possible and to facilitate the drying of particles and cells containing radioisotopes which are to be subsequently monitored with a nuclear particle detector. This latter step is to minimize beta particle absorption by the surrounding fluids, thereby increasing the sensitivity of the assay. (See Section VII,C.10).

The properties of greatest interest in selecting filters for specific applications are the pore size, the pressure flow rate relationship for the fluid to be filtered and the chemical compatibility with the fluid to be filtered.



Pore size is a measure of the largest particles which a filter can pass. Ordinarily, the size given is a nominal value and represents either a mean value of the actual pore sizes or a theoretical value for a specific material of particular dimensions when formed in a specified way, based on previous testing of that same material. In some cases two values may be used in rating pore size - nominal and absolute. In this case, the nominal value is determined in the same way as before, while the absolute value represents an estimate of the maximum particle size which it is possible for that filter to pass. While these pore size ratings must be used to select a filter for a particular application, they should be used with care, since most ratings are necessarily based on limited testing.

In general, flow rate varies with the filter material, the suspension to be filtered, the differential pressure across the filter and the pore size. Flow rate increases with pressure and pore size for the same material. The effect of filter material and composition of the fluid to be filtered is less predictable, although it can be expected that as particles are removed from suspension, plugging or partially plugging pores, the flow will be reduced. Thus the fluids with the highest particle concentrations could be expected to show the lowest flow rates.

Tests were conducted on membrane filters mounted in Swinnex type filter holders in order to estimate the pressure-flow relationships for materials which might be used in designing an AMML.



The data from these tests are presented in Tables No. 46 and 47. The data in Table 46 were taken using a filter of regenerated cellulose (rayon) with a nominal pore size of 0.20 micron. The data in Table No. 47 were based on a filter made of mixed esters of cellulose with a nominal pore size of 0.45 micron. The data were taken using distilled water and cell suspensions of varying concentrations. Tests were made for 13 and 25 mm. diameters for bath materials. The active filtration area was less than this in all cases since the filter holder grips the perimeter of the filter to provide a seal. Also in Tables No. 46 and 47, the average time in seconds to collect 10 ml. has been used to calculate mean flow rates. These have been plotted against the pressure drop for the filters tested in Figure No. 25. Curves have been drawn in connecting the points taken at different pressures "for otherwise identical conditions to aid in identifying observations. Some randomness is observed in these curves. This is to be expected due to the random error present in the gauge used to measure pressure drop and due to the possibility of randomness in the microstructure of the membranes tested.

For the small diameter filters, the slope of the pressure-flow rate curves is quite shallow suggesting that in this case, the choice of an operating pressure is not very important as long as the rupture pressure is not exceeded and there is adequate supply of pressure to insure positive flow through the rest of the system. A choice of nominal pressure from 2.5 to 15 psi would be adequate for either filter.

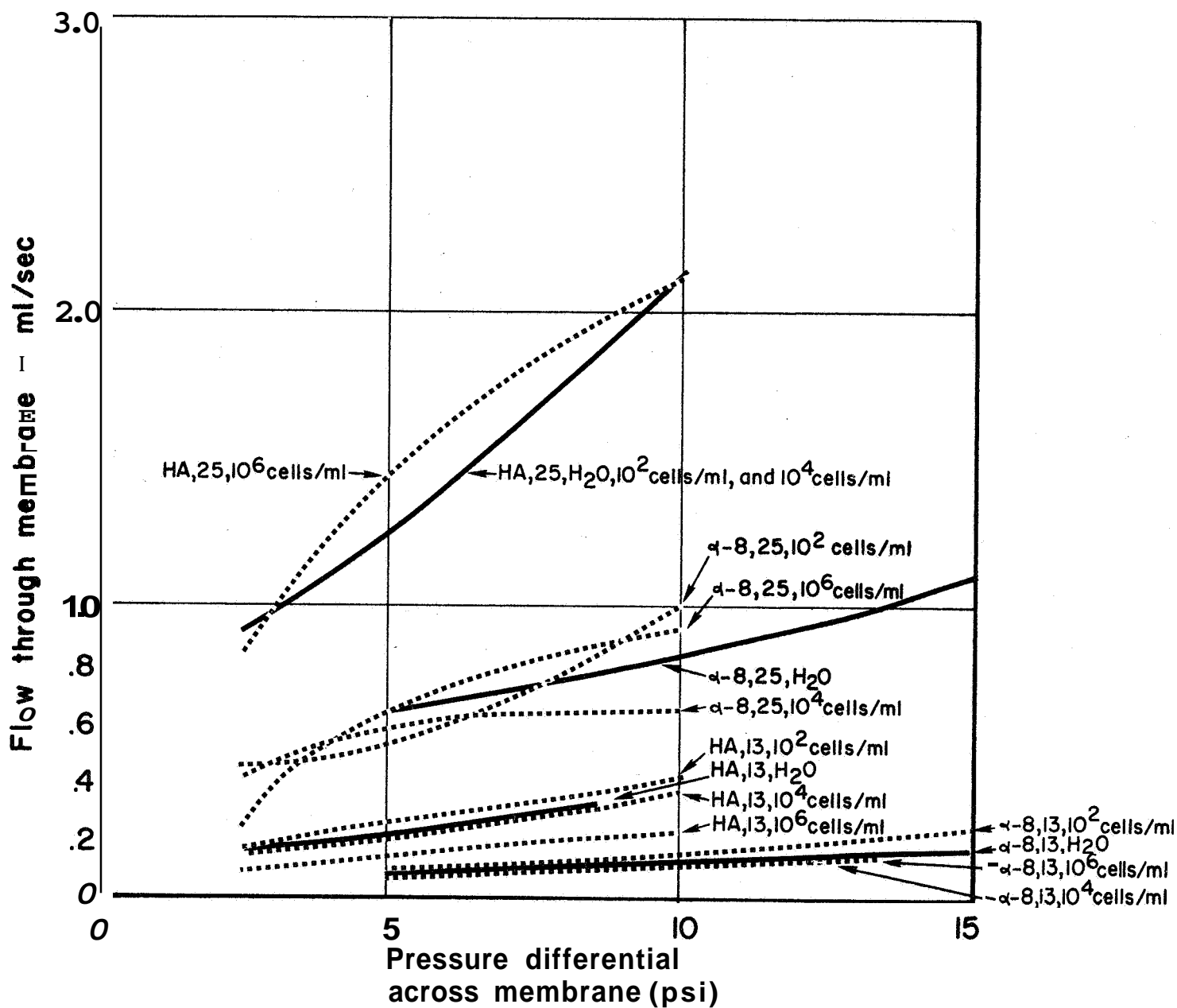


Figure No. 25 - Pressure/flow rate relationships for selected filters.

Table No. 46 - Flow rate test on α -8 membrane
filter under different pressures.

<u>FILTER</u> mm.	<u>LIQUID</u> <u>TESTED</u>	<u>PRESSURE</u> <u>(PSI)</u>	<u>FILTER</u> <u>NO.</u>	<u>TIME (SECONDS)/10 ML.</u>				<u>AVERAGE</u> <u>(9 VALUES)</u>	<u>AVERAGE</u> <u>FLOW RATE</u> ml/sec
				<u>0-10</u> ml.	<u>10-20</u> ml.	<u>20-30</u> ml.	<u>30-40</u> ml.		
13	Distilled water	5.0	1	D	100	105	104		
			2	D	108	112	113		
			3	D	105	110	110		
			Averaged		104	109	109	107	0.0934
		10	1	D	62	67	72		
			2	D	71	78	75		
			3	D	75	78	82		
			Averaged		69	74	76	73	0.137
		15	1	D	31	31	31		
			2	D	59	60	61		
			3	D	58	59	60		
			Averaged		65	69	68	54	0.185
25	Distilled water	2.5	1	D	15	16	16		
			2	D	12	13	13		
			3	D	17	18	18		
			Averaged		15	16	16	16	0.625

D = Discarded

Table No. 46 - Continued

<u>FILTER</u> mm.	<u>LIQUID</u> <u>TESTED</u>	<u>PRESSURE</u> (PSI)	<u>FILTER</u> <u>NO.</u>	<u>TIME (SECONDS)/10 ML.</u>				<u>AVERAGE</u> <u>(9 VALUES)</u>	<u>AVERAGE</u> <u>FLOW RATE</u> ml/sec
				<u>0-10</u> ml.	<u>10-20</u> ml.	<u>20-30</u> ml.	<u>30-40</u> ml.		
25	Distilled water	5.0	1	D	11	14	12		
			2	D	11	10	11		
			3	D	11	11	12		
			Averaged		11	12	12	12	0.833
		10	1	D	7	9	9		
			2	D	8	9	10		
			3	D	10	10	11		
			Averaged		8	9	10	9	1.11
		5.0	1	D	87	90	95		
			2	D	105	107	113		
			3	D	132	142	150		
			Averaged		108	113	119	113	0.0885
		10	1	D	85	87	89		
			2	D	58	61	66		
			3	D	48	51	53		
			Averaged		60	66	69	65	0.154
13	10 ² cells per ml.	15	1	D	42	43	55		
			2	D	40	40	42		
			3	D	29	30	30		
			Averaged		37	38	42	39	0.256

D = Discarded

Table No, 46 - Continued

FILTER mm.	LIQUID TESTED	PRESSURE (PSI)	FILTER NO.	TIME (SECONDS)/10 ML.				AVERAGE (9 VALUES)	AVERAGE FLOW RATE ml/sec
				0-10 ml.	10-20 ml.	20-30 ml.	30-40 ml.		
25	10^2 cells per ml.	2.5	1	D	32	34	36		
			2	D	17	17	18		
			3	D	11	11	10		
			Averaged		23	21	21	22	0.455
		5.0	1	D	10	10	10		
			2	D	37	38	39		
			3	D	9	9	10		
			Averaged		19	19	20	19	0.526
		10	1	D	14	14	14		
			2	D	7	7	7		
			3	D	6	6	7		
			Averaged		9	9	9	9	1.11
13	10^4 cells per ml.	5.0	1	D	120	120	123		
			2	D	105	107	112		
			3	D	112	113	116		
			Averaged		112	113	117	114	0.0877
		10	1	D	75	78	83		
			2	D	75	76	78		
			3	D	77	78	81		
			Averaged		76	77	81	78	0.128
		15	1	D	64	66	69		
			2	D	42	42	44		
			3	D	42	44	46		
			Averaged		49	51	53	51	0.196

D = Discarded

Table No. 46 - Continued

FILTER mm.	LIQUID TESTED	PRESSURE (PSI)	FILTER NO.	TIME (SECONDS)/10 ML.				AVERAGE (9 VALUES)	AVERAGE FLOW RATE ml/sec
				0-10 ml.	10-20 ml.	20-30 ml.	30-40 ml.		
25	10 ⁴ cells per ml.	2.5	1	D	19	20	21		
			2	D	19	17	19		
			3	D	29	30	31		
			Averaged		22	22	27	24	0.416
		5.0	1	D	16	17	19		
			2	D	15	16	16		
			3	D	18	17	19		
			Averaged		16	17	18	17	0.588
		10	1	D	21	21	21		
			2	D	14	15	15		
			3	D	12	12	12		
			Averaged		16	16	16	16	0.625
13	10 ⁶ cells per ml.	5.0	1	D	87	92	98		
			2	D	102	108	115		
			3	D	108	110	120		
			Averaged		99	103	111	104	0.0962
		10	1	D	70	75	79		
			2	D	78	83	87		
			3	D	55	60	63		
			Averaged		68	73	73	71	0.141
		35	1	D	44	46	51		
			2	D	58	61	65		
			3	D	57	60	62		
			Averaged		53	56	59	56	0.179

D = Discarded

Table No. 46 - Continued

<u>FILTER</u>	<u>LIQUID TESTED</u>	<u>PRESSURE (PSI)</u>	<u>FILTER NO.</u>	<u>TIME (SECONDS)/10 ML.</u>				<u>AVERAGE (9 VALUES)</u>	<u>AVERAGE FLOW RATE ml/sec</u>
				<u>0-10 ml.</u>	<u>10-20 ml.</u>	<u>20-30 ml.</u>	<u>30-40 ml.</u>		
25	6 10 cells per ml.	25	1	D	26	26	27		
			2	D	58	59	60		
			3	D	40	41	42		
			Averaged		41	42	43	42	0.238
	5.0		1	D	16	16	16		
			2	D	15	16	17		
			3	D	16	16	17		
			Averaged		16	16	17	16	0.625
	10		1	D	10	11	11		
			2	D	11	12	12		
			3	D	11	11	12		
			Averaged		11	11	12	11	0.910

Table No. 47 - Flow rate test on HA membrane filter
(13 mm.) under different pressures.

<u>LIQUID TESTED</u>	<u>PRESSURE (PSI)</u>	<u>FILTER NO.</u>	<u>TIME (SECONDS)/10 ML</u>				<u>AVERAGE (9 VALUES)</u>	<u>AVERAGE FLOW RATES</u> ml/sec
			<u>0-10</u> ml.	<u>10-20</u> ml.	<u>20-30</u> ml.	<u>30-40</u> ml.		
Distilled water	2.5	1	D	62.5	63	62		
		2	D	59.0	56	56		
		3	D	60.0	59	60		
		Averaged		60.5	59.3	59.3	59.7	0.167
	5.0	1	D	45	45	45		
		2	D	42.2	44	40		
		3	D	43	44	44		
		Averaged		43.4	44.3	43	43.6	0.229
	10	1	D	26	25	26		
		2	D	25	24	25		
		3	D	25	28	27		
		Averaged		25.3	25.6	26.0	25.6	0.391
10 ² cells per ml.	2.5	1	D	53	57	58		
		2	D	59	60	62		
		3	D	56	56	58		
		Averaged		56	57.6	59.3	57.6	0.173
	5.0	1	D	38	39	40		
		2	D	39	40	41		
		3	D	39	41	41		
		Ave		38.6	40	40.6	39.8	0.251

D = Discarded

Table No. 47 - Continued

LIQUID TESTED	PRESSURE (PSI)	FILTER NO.	TIME (SECONDS)/10 ML.				AVERAGE (9 VALUES)	AVERAGE FLOW RATES ml/sec
			0-10 ml.	10-20 ml.	20-30 ml.	30-40 ml.		
10^4 cells per ml.	10	1	D	26	26	26	24.4	0.410
		2	D	24	24	24		
		3	D	23	23	24		
		Averaged		24.3	24.3	24.6		
	2.5	1	D	58	58	59	58.1	0.172
		2	D	53	55	57		
		3	D	59	62	62		
		Averaged		56.7	58.3	59.3		
	5.0	1	D	39	40	42	44.6	0.224
		2	D	46	49	50		
		3	D	44	45	46		
		Averaged		43	44.7	46		
	10	1	D	30	30	31	26.7	0.374
		2	D	26	28	28		
		3	D	22	23	22		
		Averaged		26	27	27		
10^6 cells per ml.	2.5	1	D	67	84	198	106.6	0.0939
		2	D	62	77	180		
		3	D	59	72	160		
		Averaged		62.7	77.7	179.3		
	5.0	1	D	42	51	105	74.8	0.134
		2	D	40	57	90		
		3	D	43	75	170		
		Averaged		41.7	74.8	121.7		
	10	1	D	27	35	55	41.8	0.239
		2	D	31	42	73		
		3	D	26	34	53		
		Averaged		28	37	60.3		

D = Discarded

Table No. 47a - Flow rate test on HA membrane filter
(25 mm.) under different pressures.

<u>LIQUID TESTED</u>	<u>PRESSURE (PSI)</u>	<u>FILTER NO.</u>	<u>TIME (SECONDS)/10 ML.</u>				<u>AVERAGE (9 VALUES)</u>	<u>AVERAGE FLOW RATES ml/sec</u>
			<u>0-10 ml.</u>	<u>10-20 ml.</u>	<u>20-30 ml.</u>	<u>30-40 ml.</u>		
Distilled water	2.5	1	D	10	11	11		
		2	D	10	11	11		
		3	D	11	12	12		
		Averaged		10	11	11	11	0.91
	5.0	1	D	7	7	7		
		2	D	8	8	8		
		3	D	7	8	8		
		Averaged		7	8	8	8	1.25
	10	1	D	4	4.5	5		
		2	D	4.5	4.5	5		
		3	D	4	5	5		
		Averaged		4	4.5	5	4.5	2.22
10 ² cells per ml.	2.5	1	D	11	11	13		
		2	D	12	12	12		
		3	D	11	11	11		
		Averaged		11	11	12	11	0.91
	5.0	1	D	8	8	8		
		2	D	7	7	8		
		3	D	7	8	8		
		Averaged		7	8	8	8	1.25

D = Discarded

Table No, 47a - Continued

LIQUID TESTED	PRESSURE (PSI)	FILTER NO.	TIME (SECONDS)/10 ML.				AVERAGE (9 VALUES~	AVERAGE FLOW RATES ml/sec
			0-10 ml.	10-20 ml.	20-30 ml.	30-40 ml.		
10^4 cells per ml.	10	1	D	4	5	5	4.5	2.22
		2	D	4.5	4.5	4.5		
		3	D	4.5	4.5	4.5		
		Averaged		4.5	4.5	4.5		
	2.5	1	D	12	12	12	11	0.91
		2	D	11	11	12		
		3	D	10	11	11		
		Averaged		11	11	12		
	5.0	1	D	7	8	9	8	1.25
		2	D	7	8	9		
		3	D	7	8	8		
		Averaged		7	8	9		
	10	1	D	4.5	4.5	4.5	4.5	2.22
		2	D	4.5	4.5	4.5		
		3	D	4.5	4.5	4.5		
		Averaged		4.5	4.5	4.5		
10^6 cells per ml.	2.5	1	D	12	12	12	12	0.833
		2	D	12	12	12		
		3	D	12	12	12		
		Averaged		12	12	12		
	5.0	1	D	7	8	8	7	1.43
		2	D	7	7	7		
		3	D	7	7	7		
		Averaged		7	7	7		
	10	1	D	4.5	4.5	4.5	4.5	2.22
		2	D	4.5	4.5	4.5		
		3	D	4.5	4.5	4.5		
		Averaged		4.5	4.5	4.5		

D = Discarded



Biological tests have resulted in a preference for the filter made from mixed cellulose esters (HA), since it can be dried without curling and, although soluble in DMSO, it does not inhibit seriously the ATP assay sensitivity. The rayon filters were insoluble in DMSO and in any of the other reagents. They curled and cracked badly during drying and were found to be more fragile and difficult to handle at other times.

Tests have also shown the 13 mm. size to be adequate for collecting the small amount of particulate matter resulting from the assays. The use of cellulose esters (Millipore type HA) has therefore been shown to be feasible both from the biological and the engineering viewpoints. The filter size has been tentatively selected as about one-half inch - 13 mm.

Holding these filters in the instrument and removing them for read-out will constitute a major engineering design problem. Several schemes for doing this have been considered and discussed in Section D., below.

One important objective of any further engineering should be the development of a workable system for removing the filters for read-out.

6. Valving and Piping

No matter what type of fluid prime movers and filters are chosen, some remotely actuated valving will be required to control the flow. Check valves will be required at selected points to prevent reverse flow of reagents, i.e., from reaction chambers back into storage chambers. If reagents are not stored under pressure, but instead



the pressure for fluid transfer is provided after landing, then remotely actuated valves may also be needed to connect the gas pressure source to the reagent storage units before operation is possible. This latter situation is preferable since even a minute leak could be very significant over the eighthnonth storage time.

The type and number of valves will be determined by general guidelines which in every case should provide that:

1. The smallest possible number of valves be used.
2. The smallest physical size possible be used.
3. The lowest possible energy requirements for remotely actuated valves be used.
4. The construction materials must have chemical compatibility with reagents.

If electrically operated valves are selected, the energy requirements may be minimized by using magnetic latching and unlatching. The use of stainless steel or plastic construction should satisfy the chemical compatibility requirements.

Piping or tubing should be short and of the smallest diameter consistent with desired pressure drop in order to minimize the volume of entrapped fluids. The requirement for chemical compatibility exists here also, and stainless steel or plastic may be satisfactory. For a flight instrument there is the additional requirement that tubing design be able to withstand the forces encountered during acceleration periods.



In order to form some idea of the minimum tubing diameter which could be used for an **AMML** design, tests of the pressure-flow relationships through a 1 inch length of 20 ga. ^{0.023}~~(0.25)~~ in. ID) needle-tubing were performed. The resulting data are shown in Table No. 48. From this mean flow rates were calculated and plotted against pressure in Figure No. 26. The pressure flow data for filters shown in Figure No. 25, shows a flow rate of about .4 ml/sec. at 10 psi for a 13 mm. HA filter. Figure No. 26 indicates a drop of less than .5 psi for the same flow rate through the tubing. For the conceptual design of an **AMML**, it can therefore, be assumed that the only attenuation of fluid flow takes place across the filters.

7. Seals

All tubing connections will require fluid seals but at the probable operating pressures within an **AMML**, most of them pose no particular engineering problem except chemical compatibility. One interface requiring a fluid seal which could cause design problems, however, is that between the filter and the mount which holds it in use. The complications arise from the requirement for the instrument to be capable of removing the used filters for drying and read-out. Selection of the best system configuration should consider the means of effecting this seal.

8. Reaction Chambers

Generally, reaction chambers should be few in number and small in volume to minimize size, complexity, and reagent storage volumes. Where possible, reaction chambers should be integrated with other devices, i.e., reactions should take place in chambers that

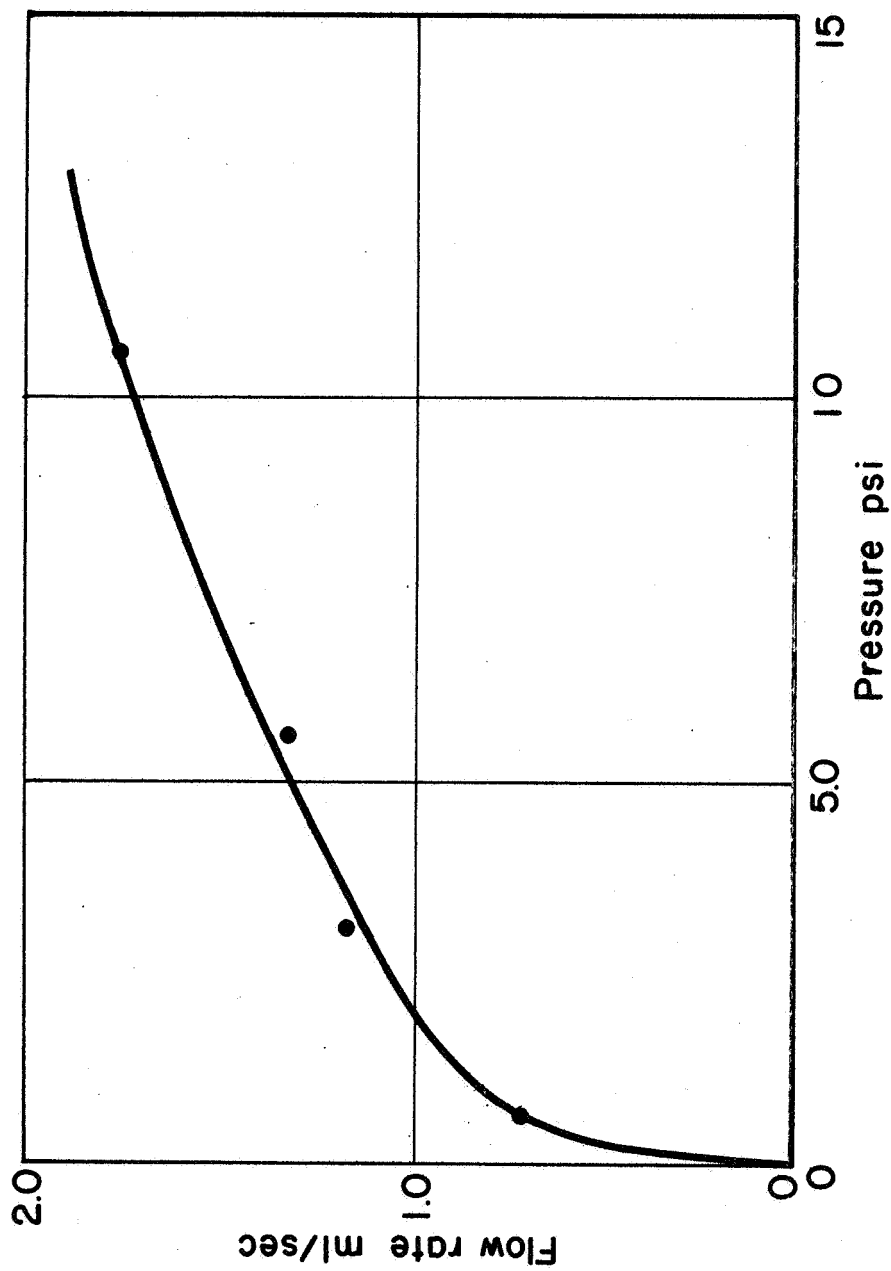


Figure No.26 - Pressure/flow relationship for water through a 20 gauge needle - one inch long

Table No. 48 - Distilled H₂O flow rate through
20-ml. syringe and needle

<u>PRESSURE</u> psi. (gage)	<u>VOLUME</u> ml.	<u>TIME</u>	<u>AVERAGE</u> <u>FLOW RATE</u> ml/sec.
0	1st 5	55 sec.	
0	2nd 5	55 sec.	
0	3rd 5	1 min. & 15 sec.	
0	4th 5	1 min. & 35 sec.	
0	20	4 min. & 40 sec.	0.0715
2.5	20	14 sec.	
2.5	20	19 sec.	
2.5	20	19 sec.	1.18
5.0	20	15.5 sec.	
5.0	20	14.5 sec.	
5.0	20	15 sec.	1.33
10.0	20	11 sec.	
10.0	20	11.5 sec.	
10.0	20	11 sec.	1.78



serve multiple functions. The construction materials must be chemically compatible with reagents. Stainless steel, or plastics such as TEE should be satisfactory.

9. Dryers

In order to get consistent and repeatable results from measurements of the radioactivity of particles on the filters, it is necessary to provide a means of drying the filters. This is due to the increased absorption of beta particles within a wet sample. The reduction in efficiency could be accounted for arithmetically, if the moisture content could be measured or controlled accurately. Observations in the laboratory have shown that the simplest way to accomplish this, however, is to reduce the moisture content to zero percent. This will provide consistent and repeatable results which correlate well with the biological parameters to be estimated. In the reduced atmospheric pressure of Mars, very little heat will need to be added to cause the water in the filters to boil off. In fact, if the operating temperature of the experiments is maintained at 26° C., the vapor pressure of water will be 25.20 mm. Hg. (Ref; 14, page D-94), well above the estimates of surface atmospheric pressure on Mars - about 5 mb, or 3.8 mm. Hg. (16) (see Section B.). Moisture remaining in filters would boil off immediately upon exposure to ambient pressure. This boiling off would occur at all temperatures above about 2.6° C. in such a pressure environment. The experiments should be conducted at a higher temperature than this to insure proper viscosity of reagents and, based on terrestrial experience, to increase the likelihood of growth of organisms collected.



Thus, it would seem that the best method of drying filters would be to provide a means of exposing them to ambient pressure just prior to read-out. The addition of small infrared sources would provide a means of speeding up the boiling process. Heaters and infrared dryers have both been tested in the laboratory and the infrared technique found to be preferable to heaters for drying filters containing cells, since the cells are raised to lower temperatures and less ATP is destroyed in the process. The concept developed here is based on the assumption that drying will be effected by exposing the filter to atmospheric pressure and that the operating temperature will be about 26° C.

10. Read-out

a, General

One of the life detection techniques considered depends on biochemical reactions which are accompanied by the emission of light. In particular, the presence of life is inferred by the presence of adenosinetriphosphate (ATP), which in turn is an absolute requirement of the luciferase-luciferin system in the firefly bioluminescence. Consequently, there is a requirement in life detection instrumentation for the detection of light.

The other life detection techniques considered depend on metabolic uptake or respiration of chemicals which may be tagged with radioactive tracers. In particular, ^{14}C and ^{35}S have been used in this program. Tracers of these elements may be detected by



application of conventional methods, including scintillation materials which in turn emit light.

The extraterrestrial application of this instrument imposes severe restrictions in size, weight, and power requirements so that the use of one single device to read-out the results of several separate methods becomes attractive. The electron multiplier phototube, or photomultiplier, has been used in prior work using ATP bioluminescence and is in widespread use as a scintillation detector. This section treats the engineering problems associated with the application of photomultipliers in an integrated instrument.

b. Bioluminescent Light Pulses (17)

The components and mechanism of the bioluminescent reaction occurring in fireflies have been well established by the excellent work of McElroy and his associates. In brief, the light emission in bioluminescence is the result of the reaction of oxygen with an oxidizable substrate (luciferin) catalyzed by an enzyme (luciferase). Luciferin must first react with ATP before it can be oxidized with light production. The total light emitted during the course of the reaction is a function of the concentrations of luciferase, luciferin, ATP, O_2 , and pyrophosphate. It has been shown that the rate-limiting step is the reaction between ATP and luciferin. Therefore, in the presence of excess luciferase, the maximum intensity is a direct function of the concentrations of luciferin and ATP. By keeping both luciferin and luciferase in excess, the maximum intensity of the emitted light is theoretically proportional to the ATP concentration,



A typical light response as measured with an oscilloscope is shown in Figure No. 27. There are two ways in which the bioluminescent response with ATP, as portrayed in the figure, can be expressed. One is by measurement of the maximum intensity of the emitted light, which after reaching this maximum value, decays exponentially. With all other factors constant, the maximum intensity is directly proportional to the concentration of ATP. The alternative manner of expressing the response is by integration of either a part or all of the total amount of light emitted, i.e., area under the light intensity curve.

From the engineering point of view, four parameters or properties of the light emission pulse are significant:

- A. Rise time
- B. Maximum amplitude
- C. Decay constant
- D. Spectral distribution

Taking the last property first, McElroy and Seliger (18), have investigated the spectral emission of several species of firefly under a variety of conditions. A typical emission spectrum is shown in Figure No. 28. The emission spectrum ranges from about 5000 to 6300 angstroms with the peak around 5600. The peak is slightly different for different species of fireflies and can be shifted by varying the pH. Although the emission peak does not coincide exactly with the

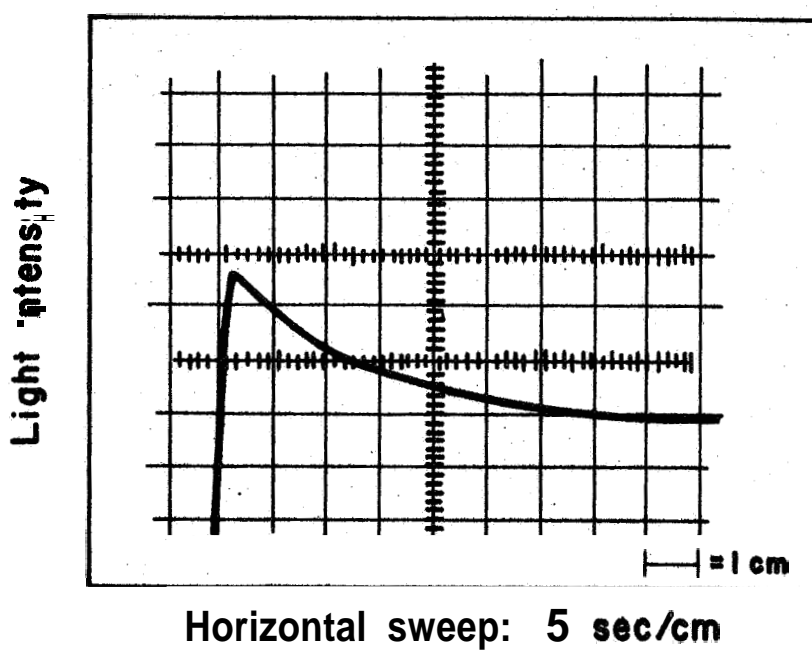


Figure No. 27– Typical response of microbial ATP in firefly bioluminescent reaction.

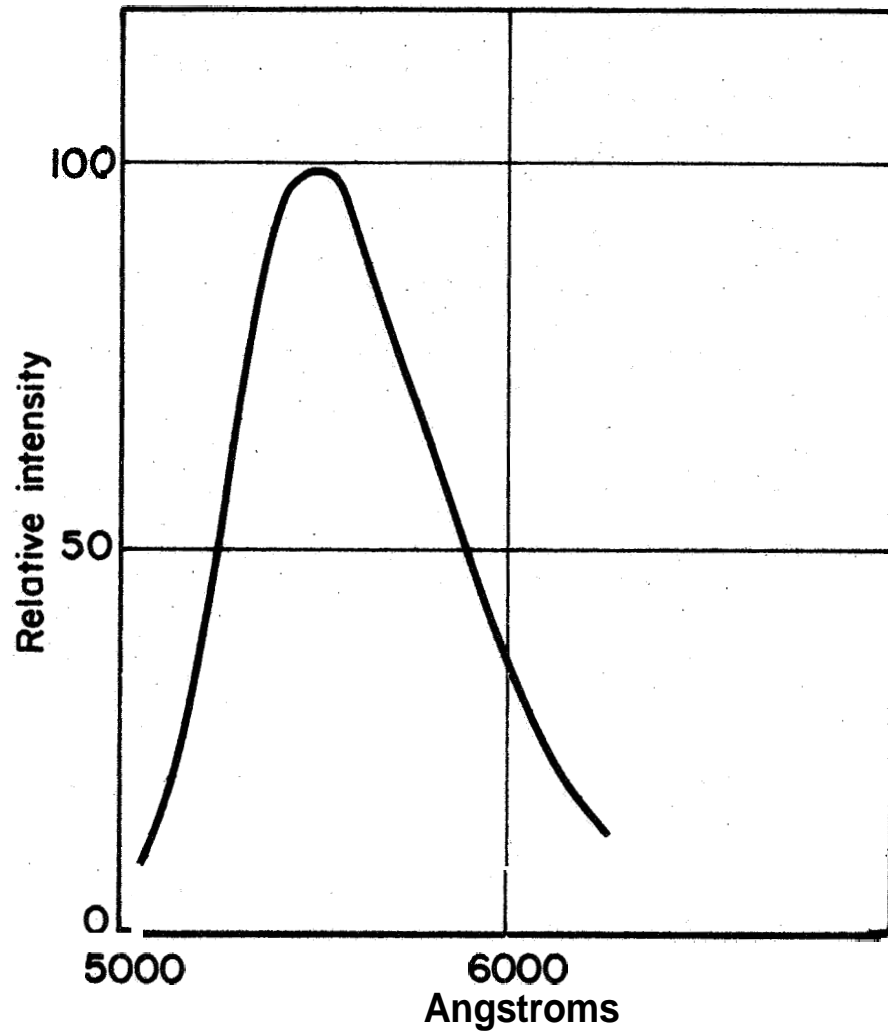


Figure No.28 -Emission spectrum of firefly
Photuris pennsylvanica



response peak of any commercially available photomultiplier, those tubes having S-20 response adequately cover the band. S-11 response also covers the spread, but at a lower relative luminous sensitivity than the S-20 .

The other three characteristics, rise time, maximum amplitude, and decay constant of the resultant current pulse are of greater significance in the design of the signal processing circuit, than in the selection of the photomultiplier itself. Once the spectral response is specified, the photomultiplier tube is selected on the basis of maximum sensitivity and minimum noise. To select the photomultiplier, an initial screening of manufacturer's data sheets for tubes having high sensitivity and low noise is made. Then a Figure of merit consisting of the ratio of the current amplification (gain) to the equivalent noise input can be calculated for each candidate tube. The tubes with the highest figure of merit are then ranked in order of lowest luminous equivalent of anode dark current. If necessary, the choice can be further limited by mechanical or economical considerations.

The length of the pulse of light from the bioluminescent reaction is of the order of seconds. The rise time has been described loosely as "instantaneous". The amplitude has been found, under certain conditions, to be proportional to the amount of ATP present; and the decay constant appears to be a function of several things, among which are the concentrations of the reactants, the speed and mode of mixing, temperature, and some not too well understood properties of the enzyme itself. Generally speaking, the light is **so** bright and lasts **so** long that a photomultiplier in any reasonable circuit cannot help but "see" it.

*

The term "sensitivity" has been used in two different senses by authors in this field. The biochemist has used the term to mean the minimum (threshold) amount of ATP (or sometimes the minimum number of cells) detectable by a particular bioluminescence instrument, while the engineer uses the term to express the ratio (slope) of the photomultiplier current per unit incident light flux.



c. SO_4 and PO_4 Uptake

As described elsewhere in this report, the metabolic uptake of SO_4 and PO_4 is measured through the use of radioactive tracer techniques. SO_4 is measured directly, using ^{35}S tag, while PO_4 is measured indirectly, since the isotope ^{32}P has a half-life of 14 days which is short relative to its practical use. Consequently, the residual ("un-uptaken") PO_4 is precipitated with triethylamine tagged with ^{14}C , ^{35}S and ^{14}C emit beta at 168 and 156 kev, respectively.

(1) Geiger-Muller Tubes

Since gaseous (ionization) detectors can be made to count beta with almost 100% efficiency, either a proportional counter or a Geiger-Muller (GM) tube could be used to determine the presence of ^{35}S and ^{14}C . Although a proportional counter would give the additional information to discriminate among events of different ionization capability (e.g., cosmic radiation), the desirability of getting large pulses favors operation above the Geiger threshold. The GM tube was the detection element for the Gulliver instrument which detects respired tagged CO_2 .

GM tubes do have window problems (19). The lower energy limit for a mica or aluminum window is about 35 kev. Thinner windows are subject to pinholes which leak gas. The gas pressures, which must be maintained at a constant value, are of the order of 2 or 3 cm. of Hg. If the pressure is too low, the GM tube will not count the lower energy betas. The gas consists of an inert component, such as argon, plus a quenching gas, often organic. Over a long period of time, the filling



gas is not only subject to leakage, but can change composition if any contamination (e.g., solder flux) is left inside the tube, **Because** of these problems, but more importantly, because of the desirability to use a common read-out system, **GM** tubes are not included in the detailed engineering concept for **AMML**.

(2) Scintillation Detectors

The advent of the photomultiplier led to a revival of the use of scintillation materials to detect energetic particles. In general, the scintillation counter has the important advantages of short resolving time, high efficiency and energy discrimination capability. The pulse height is essentially proportional to the energy of the incident particle, This property suggests that these detectors should be extremely useful for weak radioactive sources. However, inorganic crystal scintillators tend to scatter the low energy beta before the particles lose all their energy to the crystal. Organic crystal scintillators are better in this respect. Indeed, the efficiency of scintillators is usually expressed as the relative efficiency referred to anthracene. Table No. 49 lists the relative efficiency of several scintillators. Of these, **NaI(Tl)** and **CsI(Na)** are soluble in water and were not considered further. Table No. 50 lists properties of materials considered.

Tests were made with all three materials listed. In spite of its light weight, low cost, transparency, and convenient geometries possible, the relatively **low** response of the Pilot "**B**" resulted in its early elimination. **CaF₂ (Eu)** is very attractive due to the

*

Table No. 49 - Relative response of scintillating materials ■

	<u>RELATIVE PULSE HEIGHT</u>	<u>PEAK EMISSION</u> mμ.
NaI(Tl)	210	410
CsI(Na)	178	420
CaF ₂ (Eu)	105	435
Anthracene	100	440
Pilot "B" **	68	407
Stilbene	60	410

* Adapted from information supplied by manufacturers.
 ** Patented plastic scintillator manufactured by
 Pilot Chemicals, Inc., Watertown, Mass.

Table No. 50 - Properties of selected scintillating materials.

	<u>CaF₂(Eu)</u>	<u>Anthracene</u>	<u>Pilot "B"</u>
Rel pulse ht.	105	100	68
Decay const., ysec	1.1	0.032	0.002
Density g/cm ³	3.165	1.25	1.02
Ml. Wt.	78.08	178.23	
Softening Temp. ° C.	-	-	70-75
Melting Temp. ° C.	1418	216.2	-
Refractive index	1.47	1.59	1.58
Solubility:			
Water	<i>i</i>	i	i
Lower alcohols	<i>i</i>	i	i
Aromatic solvents	<i>i</i>	sl-s	s



following properties;

- a) It is very insoluble and inert and can be in direct contact with most solvents, including **DMSO**.
- b) It has a low vapor pressure and high melting point, making it attractive for space application.
- c) It has excellent transparency and a low index of refraction.
- d) It has low molecular weight and reasonably low atomic number elements, making it a good beta detector.

On the other hand, anthracene has a low time constant. In the test made, anthracene also gave a much lower background count than did $\text{CaF}_2(\text{Eu})$ (Table No. 51). As mentioned earlier, the thickness of the anthracene was of the order of 15 mils, which should be adequate for the low energy beta of ^{35}S and ^{14}C . The $\text{CaF}_2(\text{Eu})$ was much thicker - a quarter inch - which is unnecessarily thick for this purpose and contributes principally to degradation of the signal-to-noise ratio by added fluorescence and scattering. Anthracene is not as transparent nor as mechanically rugged as $\text{CaF}_2(\text{Eu})$, and it is still felt that $\text{CaF}_2(\text{Eu})$ is the preferable material. During the breadboard stage of **AMML**, the optimum thickness for the $\text{CaF}_2(\text{Eu})$ crystal should be determined to get the best balance between minimum background count and maximum energy absorption for ^{14}C and ^{35}S beta. Then the time constant of the photomultiplier load circuit should be adjusted to get the best balance between long for $\text{CaF}_2(\text{Eu})$ and short to avoid loss of counts due to closely spaced disintegrations.

Table No. 51 - Background counts with
various scintillators

<u>SCINTILLATOR</u>		<u>BACKGROUND</u> cpm *
<u>MATERIAL</u>	<u>THICKNESS</u> in.	
CaF2(Eu)	.25	141
CaF2(Eu) with Al window	.25 .005	341
Anthracene	.015	17
Pilot B	.020	98
Pilot B	.030	104
Pilot B	.050	137

* Average of three consecutive readings on Nuclear-
Chicago Model 186 scaler, sensitivity setting 10 mv.
Optical coupling with photomultiplier was air.



Tests were made to improve the optical coupling by filling the space between the scintillator crystal, $\text{CaF}_2(\text{Eu})$ in this case, and the photomultiplier with microscope immersion oil of refractive index 1.52, which very closely matched the crystal and tube. As shown in Table No. 32, a decided improvement in counting efficiency resulted. For example, compared to the Nuclear Chicago D-47, efficiency improved from 21% to 77% for a moderately active ^{14}C -triethylamine sample.

11. Timing and Control

Equipment must be provided to control the timing and sequencing of the instrument. Its functions have been depicted in Figure No. 29.

Since the series of observations for all experiments will be separated by many hours, most equipment can be turned off between samples to conserve energy. Therefore, it will be necessary to provide switching to energize equipment at the appropriate times. Wherever possible, switching should be of solid state design to reduce size, weight and power requirements. If relays are necessary to switch relatively large currents or high voltages they should be miniaturized.

Control of switching would be effected by a programmer where the master operational sequence, or program would be stored, and read. This should preferably be of solid state, electronic design to minimize weight and power requirements. If servomechanisms are needed for precise, continuously variable control of any operation, a servo control unit, also electronic, controlled by the programmer will

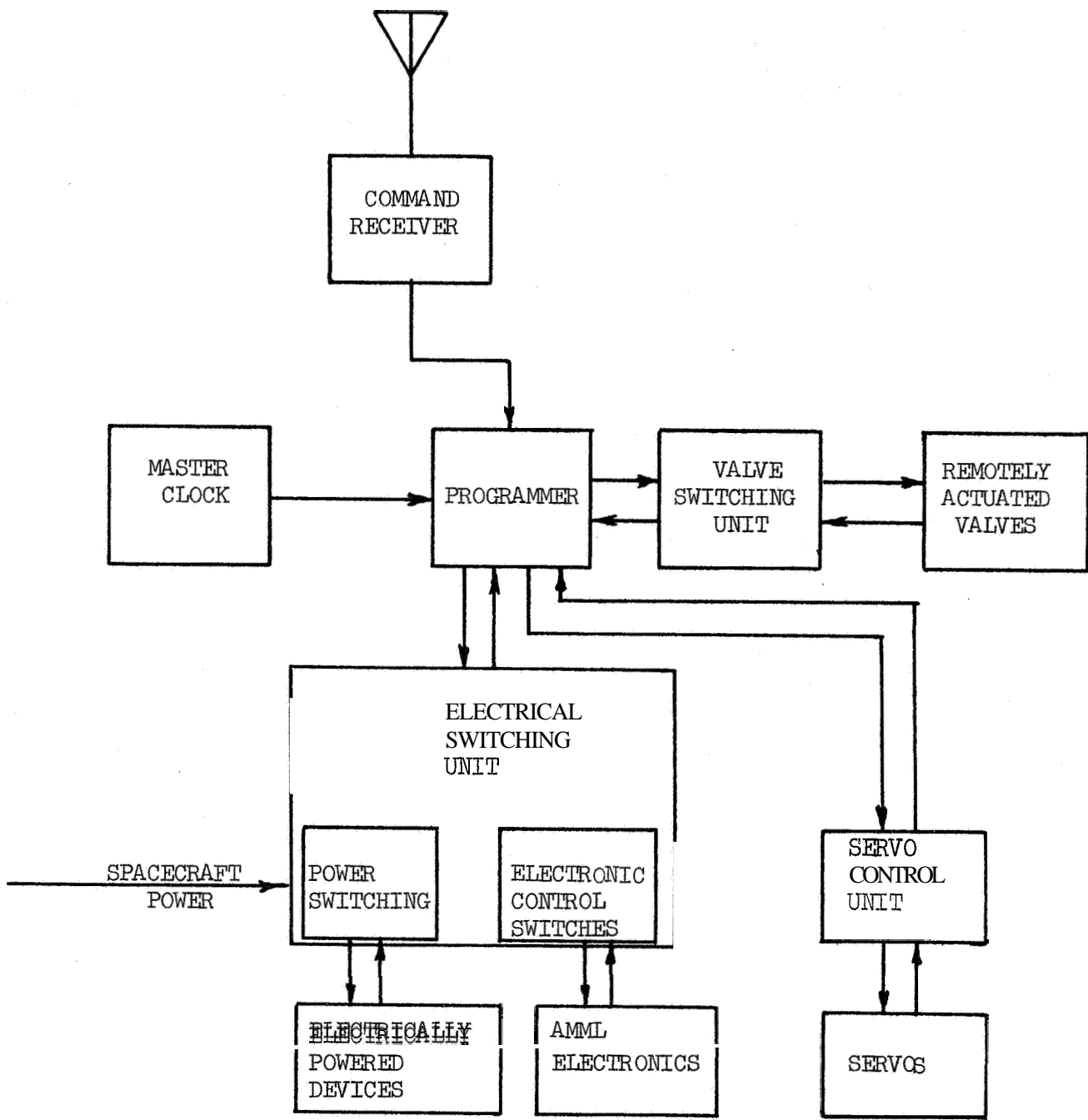


FIGURE NO. 29

Timing and Control System Block Diagram

Table No. 52 - Effect of optical coupling

	<u>D47</u> <u>cpm</u>	<u>HAZLETON INSTRUMENT</u>			
		<u>WITHOUT OIL*</u>		<u>WITH OIL</u>	
		<u>cpm</u>	<u>%</u>	<u>cpm</u>	<u>%</u>
Background **	--	141	--	283	--
Sample No. 1	969	175	18	522	54
Sample No. 2	8346	1748	21	6430	77

*

** Oil was Cargille's Type B immersion oil, refractive index 1.5150
¹⁴C on filter paper



be needed. If it is desired to modify the program during use, a command receiver link with Earth will be needed. Timing events or data must be provided to the programmer by a master clock.

Feedback, either in the form of error signals (for servos), voltages (for power switching), or microswitch closures should be used wherever possible to provide the most positive means of controlling operations.

The specific configuration of the instrument will have a large effect in determining the complexity of the timing and control subsystem. This complexity will result not only from the number of steps of the chemical process but from the operational mode in which subsequent observations are obtained. There are two general fundamentally different modes to obtain subsequent observations; to reuse reaction chambers after suitable cleaning, or to provide an unused replicate set of chambers for each observation. The first method would result in a considerably simpler, smaller, and less massive instrument. If the latter method is used, due to difficulty in cleaning or other considerations, the instrument will be more complex and larger and the timing and control system will also be more extensive. Both alternatives will be discussed further in Section D.

12. Signal Processing, Data Storage and Telemetry

Data obtained from the observations must be processed, possibly stored, and telemetered to Earth. Signal processing circuitry must be provided for AMML equipment but data storage and telemetry may



be shared with other experiments on board by suitable multiplexing.

Data storage helps to prevent the loss of data and allows the use of narrow band telemetry equipment, thus lowering the intelligence frequencies which must be transmitted.

These problems are beyond the scope of this contract, but it should be kept in mind that the output signals must be compatible with these requirements.

13. Other System Requirements

Other requirements of an AML will be summarized briefly. At present there are no quantitative limits set on these requirements and they can only be discussed very generally at this point. These limits can be established after the spacecraft and booster detailed capabilities are known.

With the possible exception of the timing unit, it will not be necessary to operate any of the AML equipment during the eight-month cruise. Periodic operation may be desirable from the reliability viewpoint, but not for data collection. Thus the AML equipment might be carried through most of the journey at a low temperature. If one end of a spacecraft axis were always oriented toward the sun, as in Mariner IV, it would be possible to maintain such a low temperature by pointing a black body, which acts as a heat sink for the AML equipment, into empty space, thus reducing the power required for environmental control during the journey there. This would have the additional advantage of inhibiting the growth of any microorganisms which survived the sterilization procedure before a launch.



It is planned to conduct the experiment at about 26°C. , since this temperature lies within the diurnal cycle on much of the Martian surface and any organisms found should be able to withstand it. Furthermore, this temperature is on the high side of the diurnal cycle and since terrestrial organisms grow best in a range of the higher temperatures which they can withstand, it has been assumed that on Mars also, this temperature may be conducive to growth.

In order to bring the instrument and reagents to the operating temperature, environmental control equipment must be provided and activated well in advance of landing, possibly several days. The enzyme storage chamber must still be kept cold (less than -10°C.), however. The temperature control equipment must also operate continuously during the hours in which the **AMML** operates. Power requirements are at a peak for short periods (perhaps 10 minutes), while an observation is being made and then reduced to that used by temperature controls, timing and control equipment which must be kept energized to control operations. for intervals of several hours. Telemetry and signal processing will probably be energized only at specific times, **also** to reduce power requirements.

There is also a requirement for reliability engineering to be performed when an **AMML** is built. The probability of failure **of** individual components must be kept at a minimum and the interconnection of components such as the reliability of the system is preserved.



Reliability goals should be established quantitatively by means of statistical probabilities in accordance with current technology. The probability of successful operation of a life detection instrument in an unknown ecology may already be quite low without further degrading it with a high probability of system failure. At this point, reliability can only be considered qualitatively by assuming that the simplest systems are usually the most reliable.

14. Sterilization

The final requirement to be mentioned is that of sterilization. The Committee on Space Research (COSPAR) of the International Council of Scientific Union has recommended (20)

".....that the basic probability of 1×10^{-3} that a planet will be contaminated during the period of biological exploration.....be accepted as the guiding criterion for the exploration of Mars..... "

While research in spacecraft sterilization methods continues, it has been learned to date that assembly in a sterile laboratory followed by a heat cycle of 24.5 hours at 125°C . will meet the above criterion for sterilization (Ref: 20,p.189).

The construction materials and the chemical reagents used in an AMML must therefore, be able to withstand a temperature of at least 125°C ., for what amounts to an indefinite period or some equivalent means of sterilization found. It will probably be possible to sterilize the assembled AMML, less reagents, completely with heat as described, although extreme care must be used in selecting



electrical and electronic components and fluid seals, such as O-rings. It will probably not be possible to use such a sterilization technique on certain of the chemical reagents, particularly the enzyme.

D. System Configurations

1. The Basic Module for One Operation

The purpose of this section is to present hypothetical system designs, describe their sequencing through one operation and evaluate them. One operation is defined **as** the processing of one sample to determine SO_4 uptake, PO_4 uptake and ATP content. Each time the test is made, this operation must be performed at least twice, once for a sample with incubating medium and once for a control sample with poisoned medium. The experiment may consist of replicate tests at stated time intervals, for a total of, say, 144 hours. (The intervals need not be equal). Such an experiment would require seven tests, i.e., an initial one at time zero and six replicates, for a total of fourteen operations. Two fundamentally different concepts suggest themselves, each however, based upon the application of a basic module which will perform the "one operation":

- a. An apparatus consisting of an assemblage of non-reusable basic modules equal in number to the number of times the operation is to be repeated. (14 in the above example) , **or:**
- b. An apparatus consisting of two basic modules, one for test and one for control with provision for



reusing them.

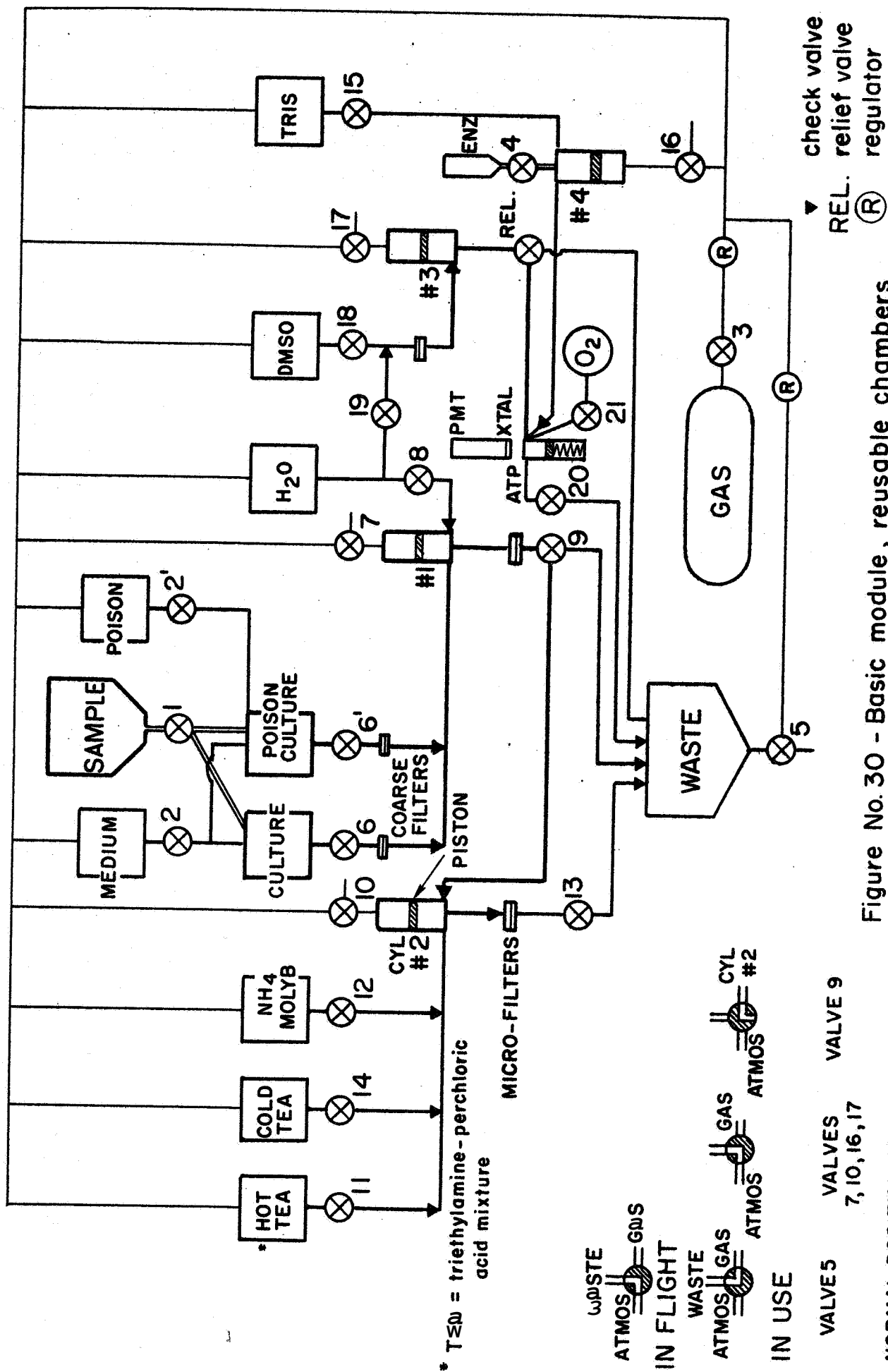
The choice is examined later; first the basic module itself is considered, addressing the functions which need be performed rather than specific hardware. The basic module must :

- a. Accept a sample
- b. Filter out the cells
- c. Determine directly the SO_4 taken up by the cells
- d. Determine the presence of ATP in the cells
- e. Determine indirectly the PO_4 taken up by the cells by measuring the amount left in the filtrate
- f. Present the above data in a form amenable to transmission to Earth.

2. Reusable Reaction Chamber

A schematic diagram for a basic module of an instrument with reusable chambers is shown in Figure No. 30. Measured volumes of fluids are determined by positive displacement of pistons rather than by flow rate through valves and, in general, pistons are motivated by a pressurized inert gas rather than by electromechanical means. These two general specifications contribute to simplicity and reliability, and minimize electrical power requirements.

The general scheme of operation is as follows: A sample is mixed with a metabolic nutrient broth in the sample tank and allowed to incubate. At predetermined time intervals, an aliquot of fixed volume is drawn off into cylinder 1. This volume is then filtered to





remove the cells, the filtrate going into cylinder 2, where it is mixed with radioactively tagged triethylamine (hot TEA), and an ammonium molybdate-perchloric acid mixture to precipitate the remaining PO_4 . The precipitate is filtered out and washed with untagged triethylamine (cold TEA). The SO_4 and precipitated PO_4 filters are then dried and read by a scintillation counter to get data to determine SO_4 and PO_4 uptake.

Meanwhile, a tablet or pill of the enzyme luciferase has been dissolved in Tris buffer in the ATP chamber and allowed to stand to reduce the inherent light. After SO_4 uptake is read-out, the SO_4 filter is dissolved by DMSO to extract ATP. The solution is injected into the ATP chamber and the resulting bioluminescence read by the photomultiplier.

Removal of the filters for read-out and replacement for subsequent observations would be facilitated by mounting the filters in a tape and transporting them through the instrument with a tape drive system (see Figure No. 31). The body of the instrument would be manufactured in two parts which could be clamped shut to seal the filter in or opened to allow its motion. Although the composition and structure of the tape, i.e., tape made of filter material or filter material mounted in a tape of some other material, would require further study, no insurmountable problems are envisioned in developing such a filtering system. In order to reuse the chambers the instrument is flushed after each use. Laboratory tests have shown two flushes with distilled water to be sufficient.

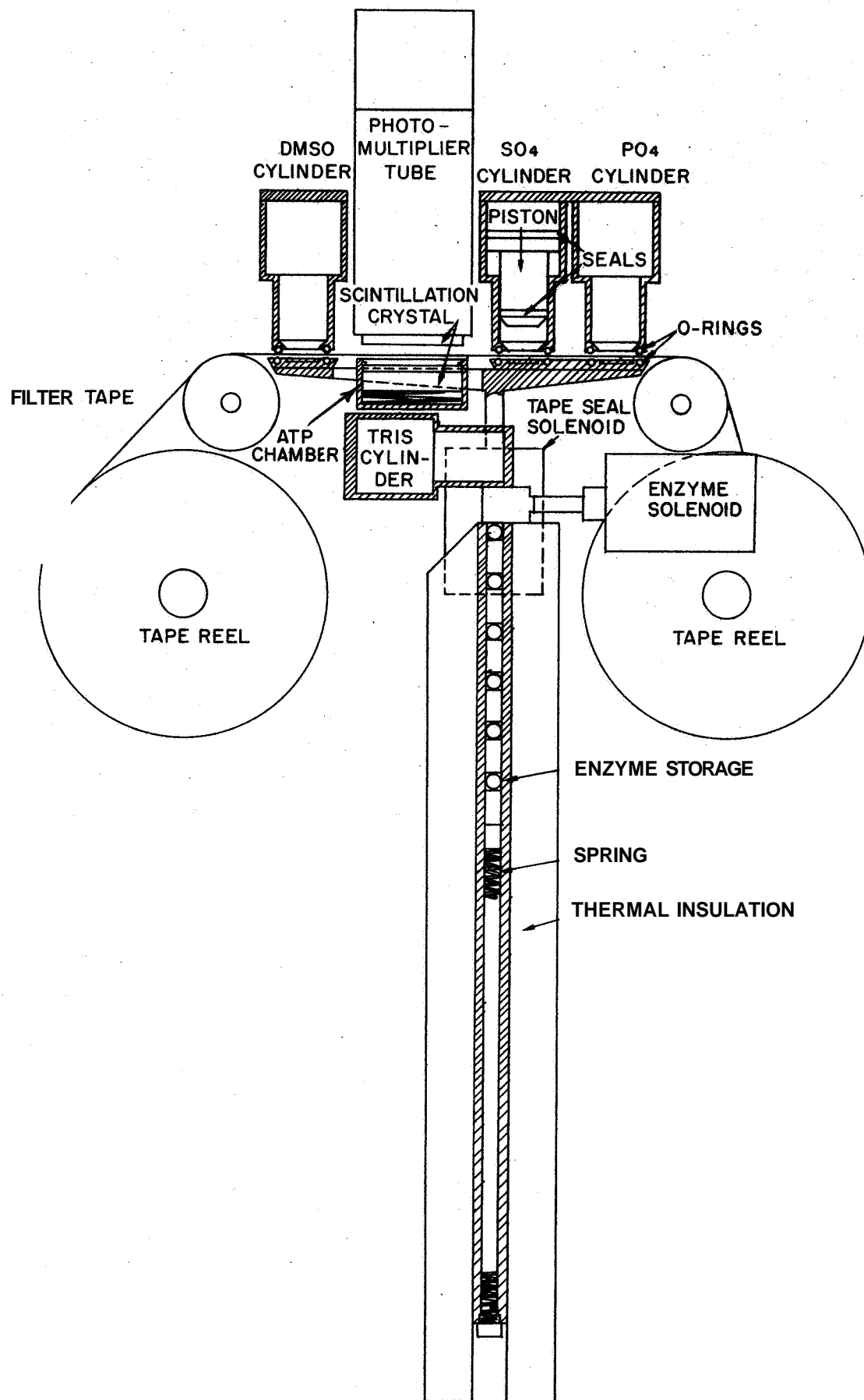


Figure No. 31 - Concept for tape transported filters



The step-by-step operating sequence of this scheme follows :

OPERATING SEQUENCE

Step	<u>Action</u>	<u>Time</u>	
		<u>Min.</u>	<u>Sec.</u>
1	Inject enzyme tablet into ATP reaction chamber.	0	
	Open Tris supply valve 15, filling Tris cylinder.		
	Turn on power supply.		
	Open sample tank supply port 1, allowing sifted soil particles to enter sample tank.	*	
2	Operate normally vented 3-way valve 16, injecting measured volume of Tris into ATP chamber.	0 - 5	
	Close sample tank supply port 1.	0 - 5	
	Open broth supply valve 2.	*	
	Admit O ₂ by valve 21.	*	
	Operate normally vented 3-way valve 5 to pressurized drain tank.	a - 5	
	Vent 3-way valves 7 and 10.		
3	Open sample tank drain valve 6, allowing sample aliquot to pass through coarse filter and into sample cylinder.	23 -	0
4.	Close sample tank drain valve 6.	23 -	10
	Operate 3-way valve 7,		
	Operate valve 9 to connect cylinders 1 and 2.		

* One time operation.



OPERATING SEQUENCE

Step	Action	Time	
		Min.	Sec.
5	Open H ₂ O valve 8, filling cylinder #1 with H ₂ O. Vent valve 7.	23	- 20
	Open Hot TEA valve 11, allowing TEA to enter cylinder #2 until piston follower reaches microswitch #1 position. Vent valve 10		
6	Close H ₂ O valve 8 and operate valve 9 to drain.	23	- 30
	Open ammonium molybdate mixture valve 12, allowing piston in cylinder #2 to move to detent.		
7	Operate 3-way valve 10, injecting through filter catching precipitate and draining filtrate into pressurized drain tank.	23	- 40
	Operate 3-way valve 7, injecting H ₂ O through filters.		
8	Open cold TEA valve 14, allowing piston to move to microswitch thereby closing cold TEA valve. Immediately afterward operate valve 12, to admit ammonium molybdate. Vent valve 10.	23	- 50
9	Inject cold TEA through filter valves 10 and 13 to	23	- 55
10	Close drain tank input valve 13.	24	- 0
	Open filter clamps - allow filters to dry.		
11	Read filters.	25	- 0
12	Open DMSO valve 18, allowing cylinder #3 to fill through filter containing organisms, thus extracting ATP. Vent valve 17.	30	- 0



OPERATING SEQUENCE

Step	<u>Action</u>	<u>Time</u>	
		<u>Min.</u>	<u>Sec.</u>
12	Read inherent light level of enzyme mixture.	30	- 0
13	Close DMSO valve 18. Operate 3-way valve 17, injecting the measured volume of DMSO with extracted ATP into reaction chamber. Read gross response.	30	- 5
14	Drain ATP chamber.	30	- 15
15	Vent 3-way valve 17, allowing pressure to bleed off. Open H ₂ O valves, 8 and 19, allowing H ₂ O to fill DMSO cylinder #3 and sulfate cylinder #1.	30	- 25
16	Flush cylinders 1 and 3 with water operating valves 7, 9, 10, 17, and 20.	30	- 28
17	Flush cylinder 2 by operating valves 10 and 13.	30	- 32
18-19-20	Repeat steps 15-16-17. Vent valve 5.	30	- 36
21	Close valves 13 and 20.	30	- 47
22	Return all valves to normally deenergized positions. Wait required interval for next sample then begin next sequence.	33	- 0

The above sequence is presented in chart form in Figure No. 32.

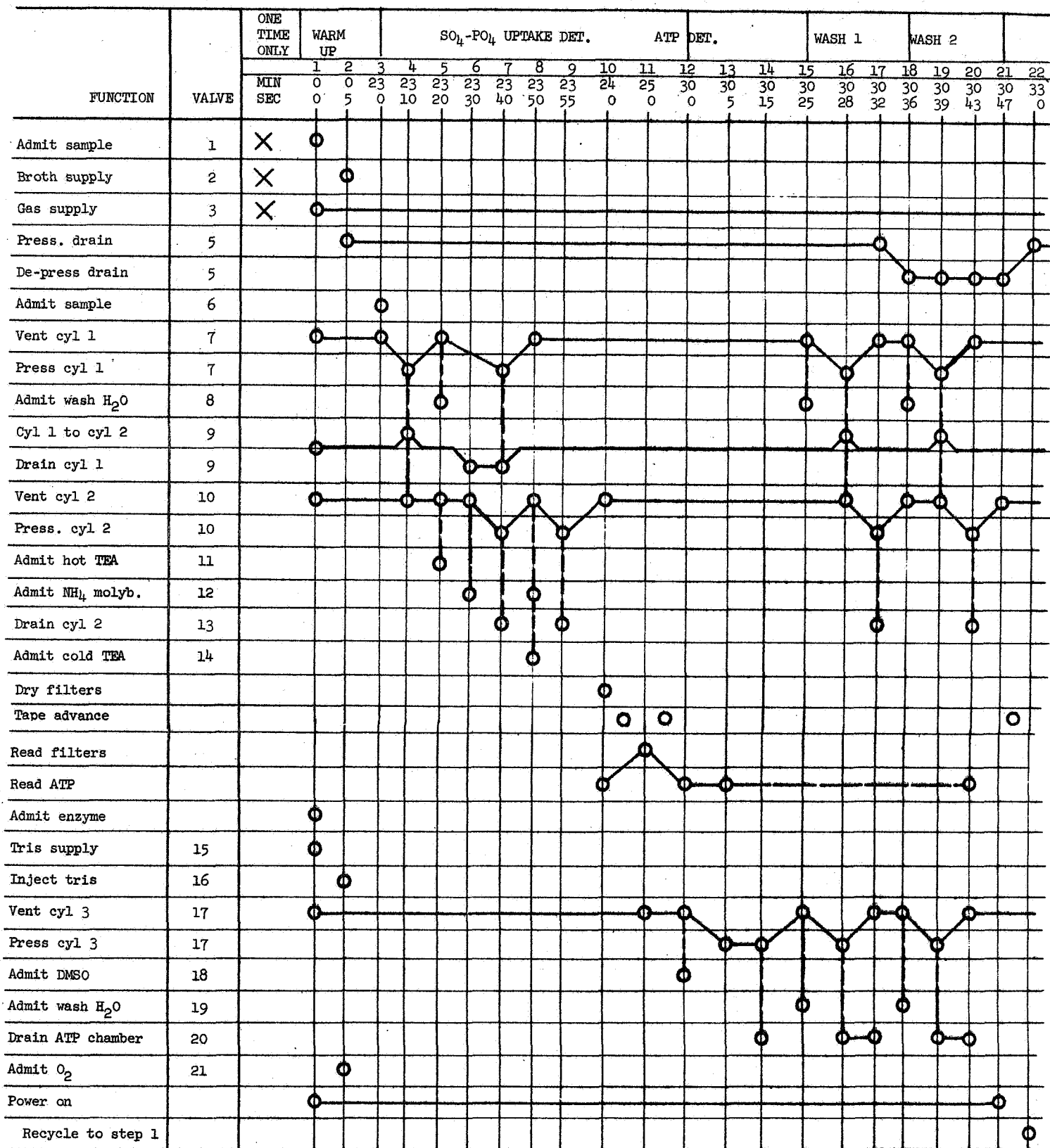


Figure No. 32 - Operational Sequence, Reusable Chambers



3. Non reusable Reaction Chambers

Of the great variety of configurations in which a basic module that is not reusable *may* be laid out, three are discussed as practically-attainable systems: A grid, a disc or wheel, and a 'carousel' arrangement, (see Figures No. 33, 34, and 35).

In each case, the design is to an objective of an initial test plus six replicate tests for a total of fourteen operations. Since the basic module consists of three read-out positions (SO_4 , PO_4 and ATP), each arrangement consists of a total of 42 positions.

In all cases, the basic module will not require the water-washes but will require additional piping and valving to all the modules and will require relative motion between the detector and the greater number of read-out positions. The increased number of valves, in particular, adds significantly to the size and weight. Another disadvantage of the array type of instrument is the requirement to distribute equal sample volumes to all SO_4 cylinders.

All the matrix configurations have problems of fluid transfer, i.e., how to push and meter fluids under all conditions of attitude and degrees of gravity, and how to overcome the vapor pressures associated with the reagents at, say, 26°C ., which are high compared to the Martian atmosphere.

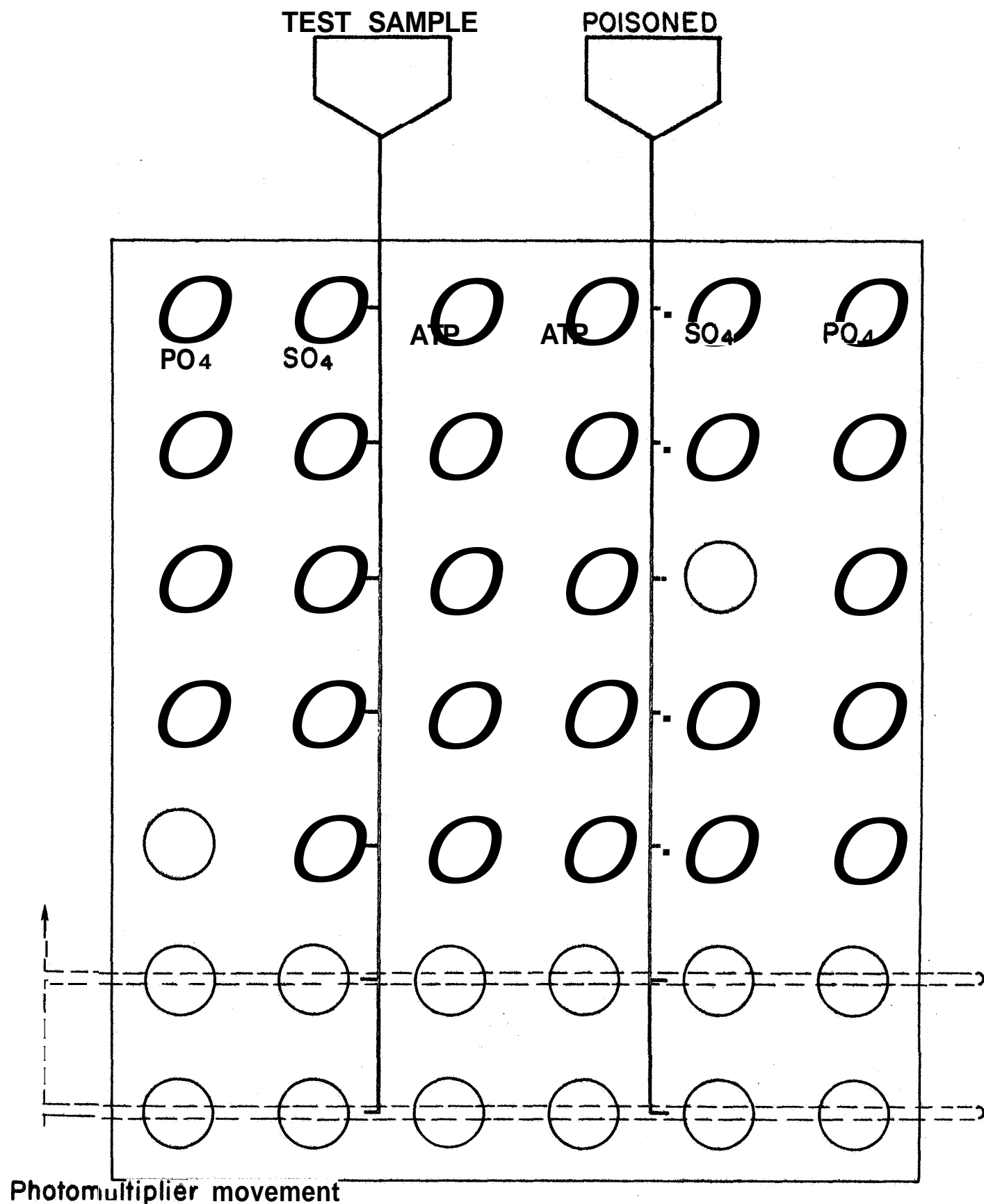
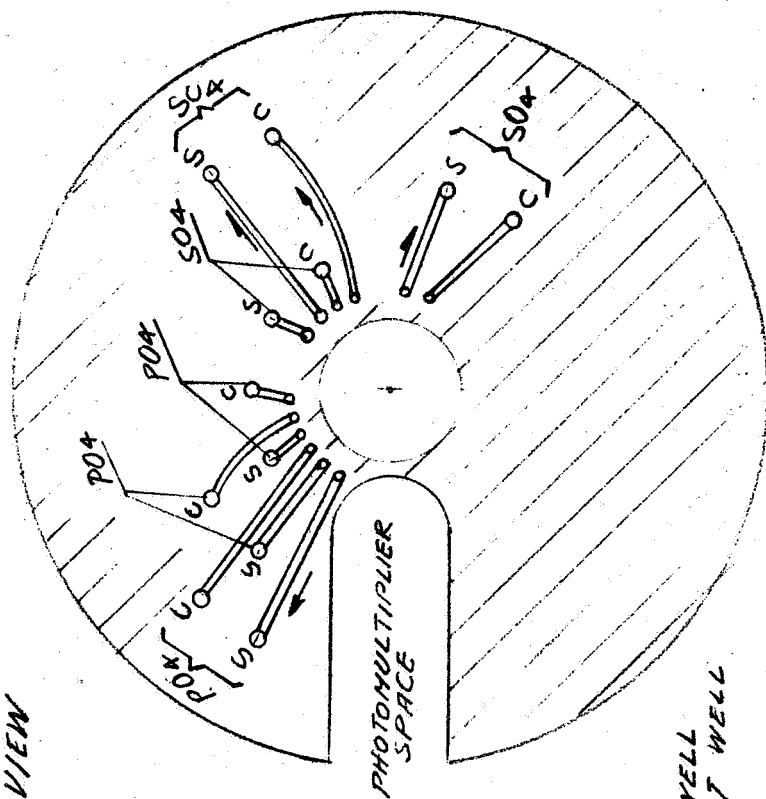
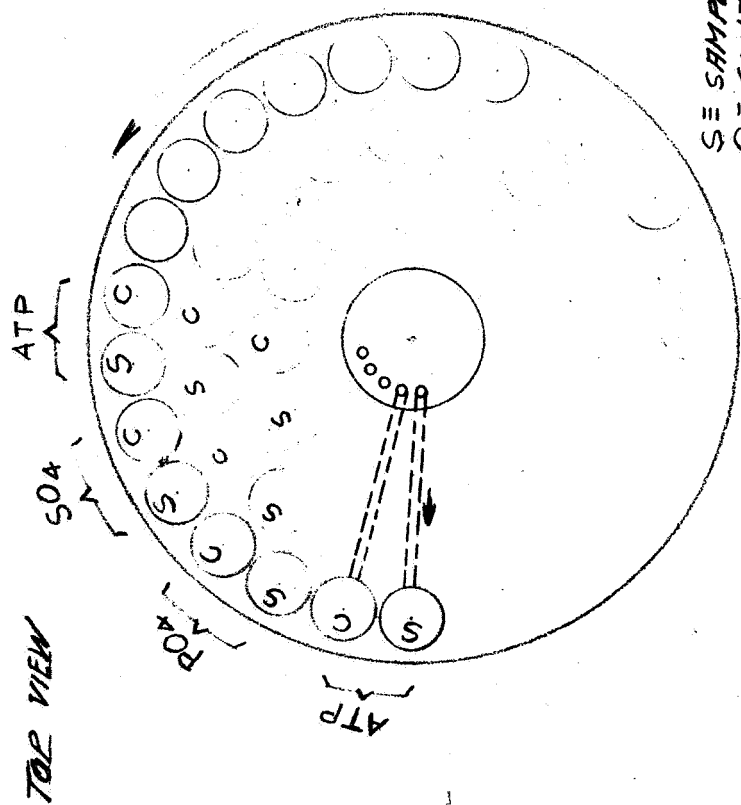
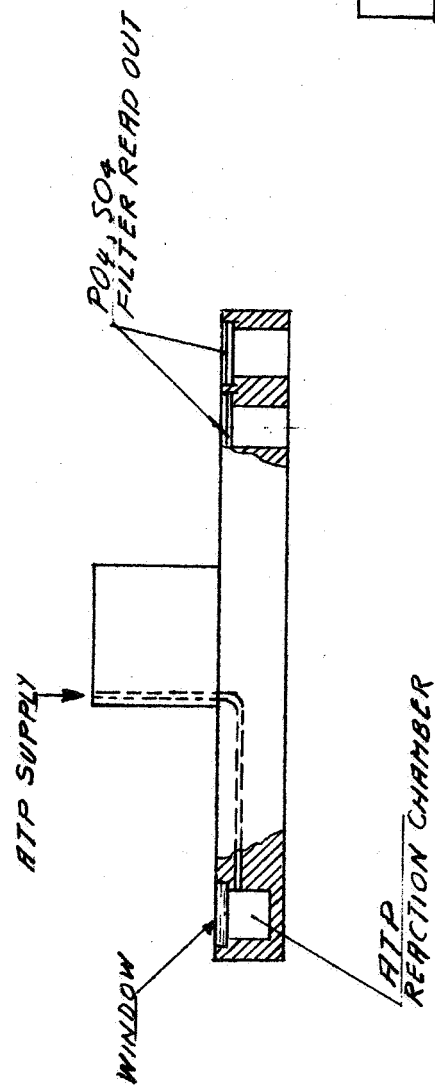


Figure No.33 - Grid concept

SECTION
TOP VIEW



S = SAMPLE READ OUT WELL
C = CONTROL READ OUT WELL



READ OUT WELLS -
MOVING DISC

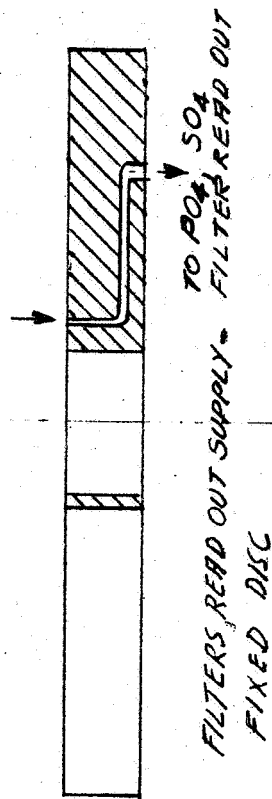


FIGURE NO.34 - DISC CONCEPT

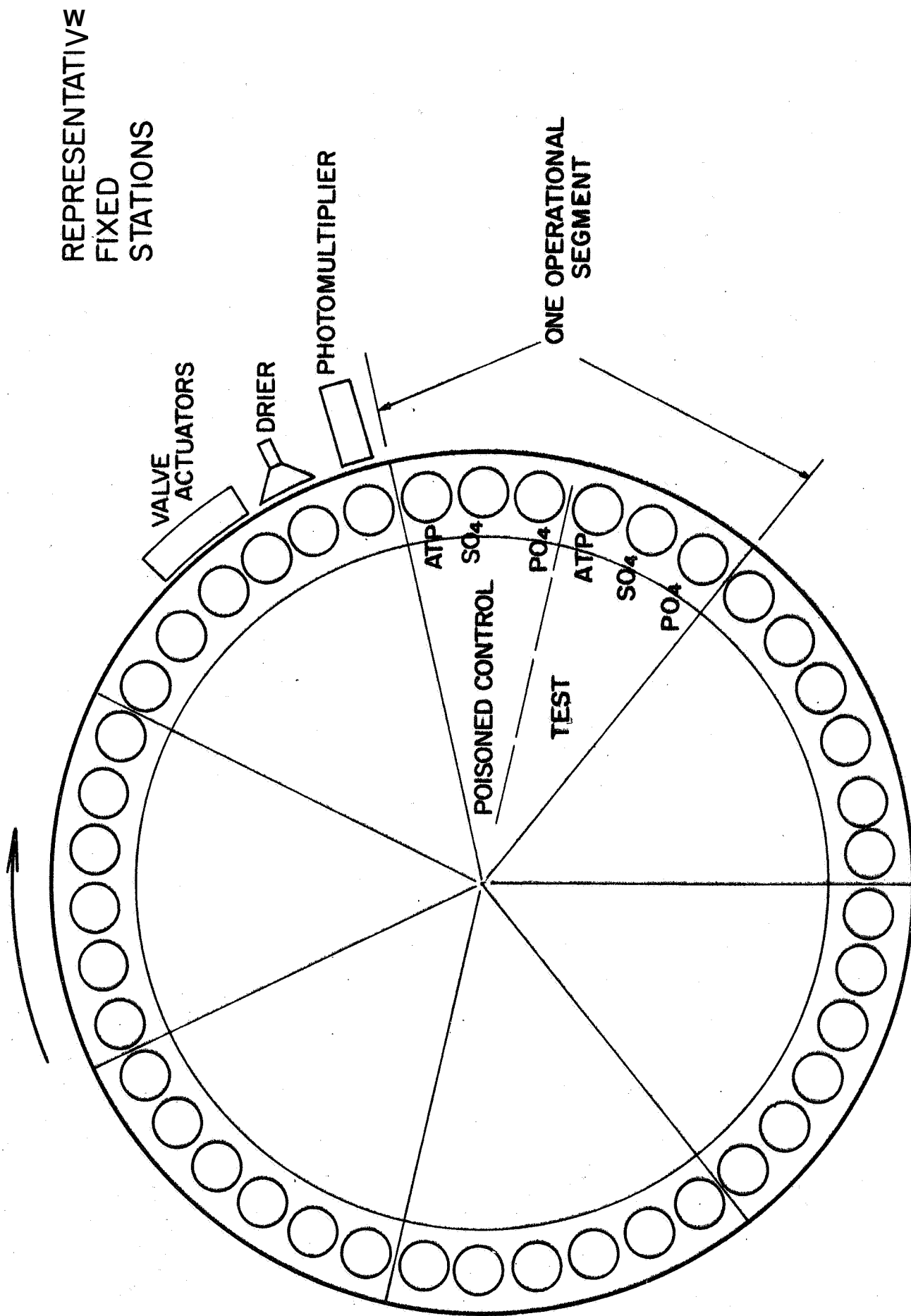


Figure No. 35 - CAROUSEL CONCEPT



In order to find some way to avoid having to open the instrument "sandwich fashion", whether grid, wheel, or carousel, some thought was also given to designing the cylinders so that the filter moved with the piston as in Figure No. 36. This complicates the design of the piston cylinder, and valving but could be developed should it be decided to reuse reaction chambers.

In this design, the drive piston would probably need to be double-acting, since it must move up to force the filtrate through the membrane, then down to eject the filtrate from the cylinder, and back up to place the radioactive samples near the scintillator. A check valve in the stem prevents reverse flow. The 3-way valves used to control piston movement must be replaced by a 4-way valves. However, there is still a great deal of similarity between systems and in their sequences of operations (See Figure No. 37 and No. 38).

Since chambers are not reused, the flush sequence is omitted and it is unnecessary to drain the reaction chambers at the end of the sequence. While a drain sequence has been shown in order to empty the wastetank, it contains only the wash water and cold TEA used to rinse radioactive particles from the cells and precipitates prior to being monitored. If the wastetank had sufficient capacity, it need not be emptied, thus saving a little time and energy. A pressurized wastetank is still retained as in the previous design to prevent flash evaporation of filtrates leaving undesired salts on the filter surfaces. Also in this case, there is no requirement to advance or move filters, although the FM tube must be advanced. As

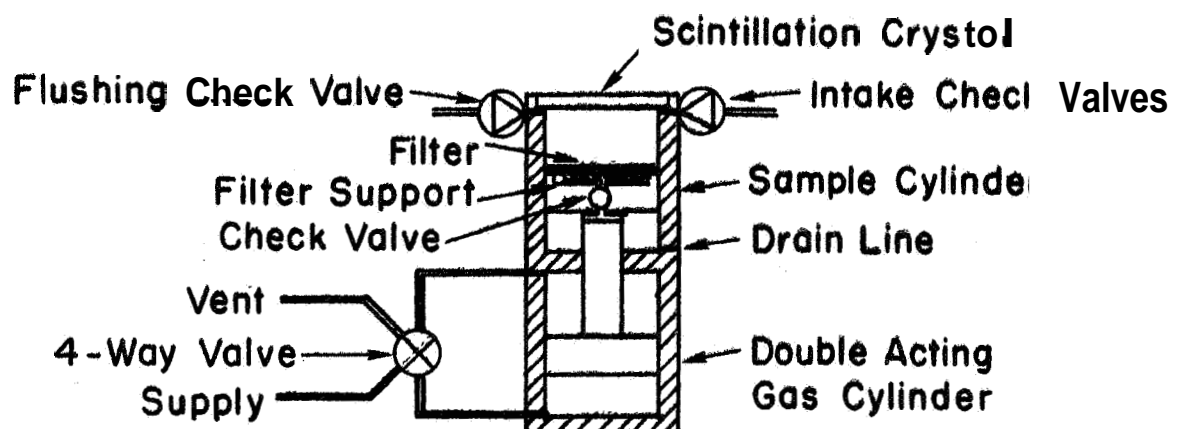


Figure No.36 - Syringe with internally mounted filter

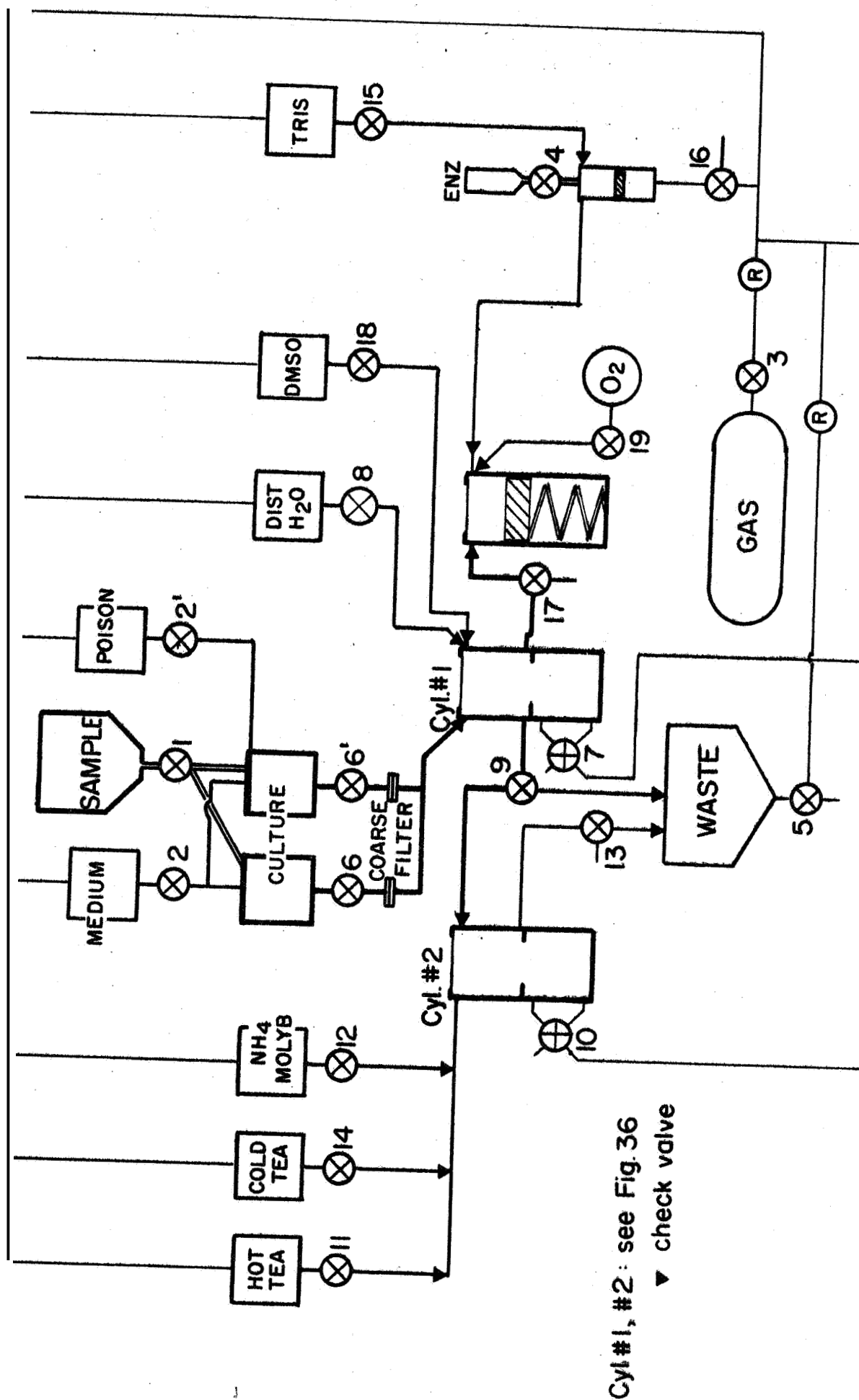


Figure No.37 - Basic module, non-reusable chambers

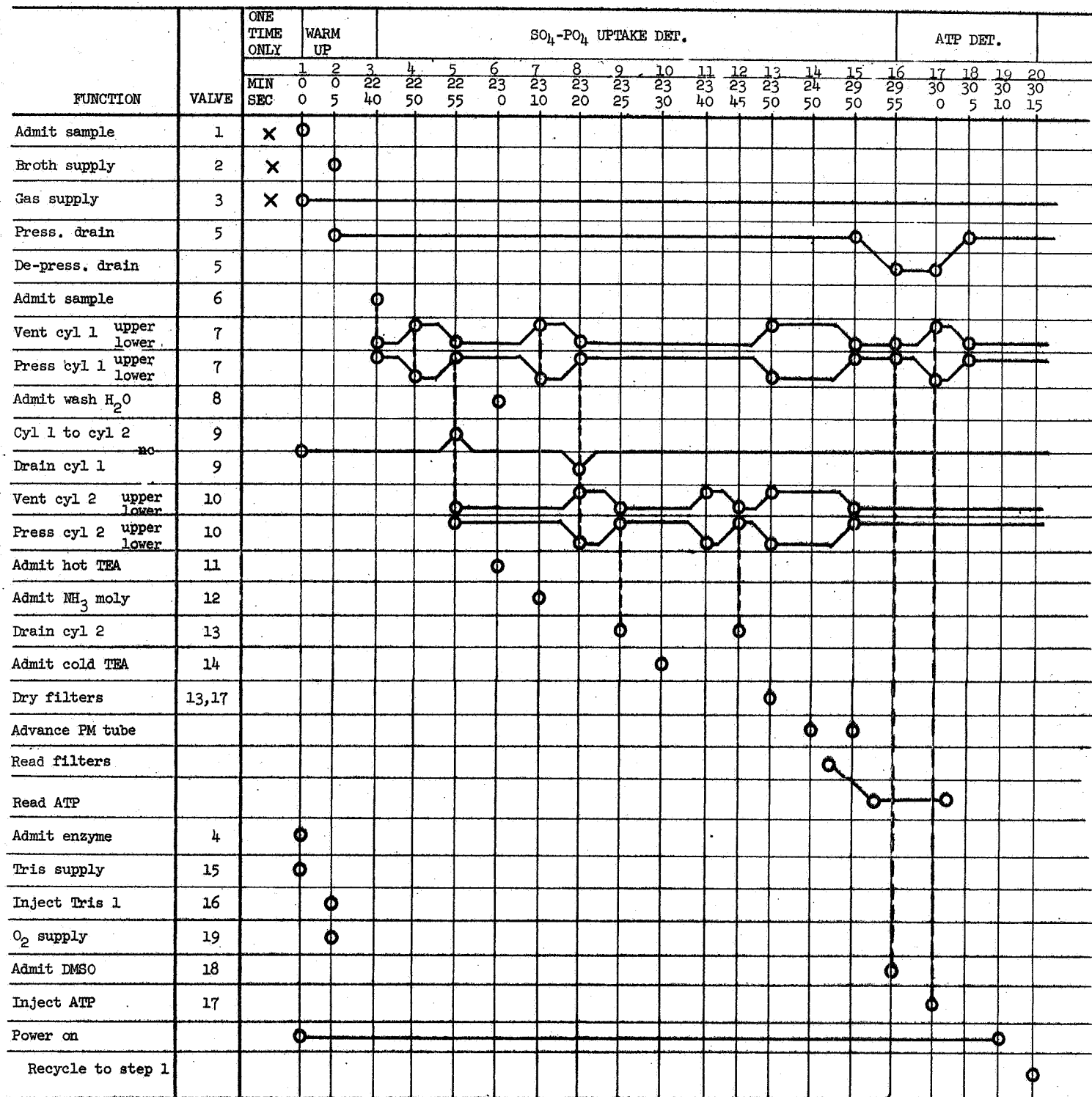


Figure No. 38 - Operational Sequence, Throw-Away Chambers



predicted, the fluid transfer sequence is more involved and slightly longer but the total power-on time is about the same (ca 30 min.) and the number of remotely operated valves is about the same.

While this piston/cylinder design, i.e., with scintillation crystal as the cylinder head could also be used on grid, wheel and carousel configurations, the carousel is preferable, since motion at the PM tube detector could be limited to one or at most, two degrees of freedom.

It should also be noted that provisions must be made to Vent the sample side of the piston in order to dry the filters. This can be done by making the drain valves (13 and 17) 3-way instead of 2-way valves, thereby adding a vent position in their sequence.

4. Selection of a Design

Of the four system designs considered, the version with reusable reaction chambers and replaceable filters mounted in a tape seems the most practical for development. It requires the least hardware, particularly moving hardware, of the four, with the same volume of reagents required. The only difference in chemical storage requirements is to carry additional distilled water to allow flushing between observations. Choosing this approach should therefore, result in lower weight, smaller size, and higher reliability than any of the other three,

Electrical power requirements and requirements for power from other sources should be about the same for all four designs. All



of the configurations would require continued development at similar levels and none is considered beyond the state of the art electromechanically. Support equipment required, i., e., signal processing, control logic, storage chambers, etc., ~~can~~ be expected to be greatest in the system with most redundancy. This also points to the advisability of the reusable chamber design. It is, therefore, recommended that the first configuration described be considered for further development.

5. Estimate of Weight, Size, and Power Required

An estimate of the weight, size, and power required for an AML of the reusable chamber type has been compiled in Table No. 53. To prepare this list the required components were listed and their individual weights, sizes, and power requirements estimated. The sum was then assumed to be a measure of the requirements for the system. Individual requirements were calculated or estimated from manufacturer's literature on similar equipment. It is anticipated that these estimates are conservative, since flight hardware undergoes extensive product engineering to minimize its needs. Two figures were developed, a maximum and a minimum for each parameter. The maximum figure assumed valves with individual operating solenoids would be used for remotely actuated valving. The minimum figure assumed a valve block with one motor or solenoid to operate all 23 valves could be developed. The latter course is definitely possible. There is no requirement to actuate two or more valves simultaneously, although some of the valve operations may be closely spaced in time; perhaps as little as 5-10 milliseconds.

Table No. 53 - Equipment List

COMPONENT	NO. UNITS	SIZE		WEIGHT		POWER		REMARKS
		PER UNIT in ³	TOTAL in ³	PER UNIT oz.	TOTAL oz.	PER UNIT w.	TOTAL w.	
1 Valves, 2-way, 2 position, momentary contact (1, 1', 2, 2', 3, 6, 6', 8, 11, 12, 13, 14, 15, 18, 19, 20)	16	2 4	54 4	5	80	5		While the individual power requirements for each valve equals 5 to 7 w. there is no requirement to operate any two simultaneously. If control logic is provided to cause sequential switching, power requirements can be kept to under 10 w. except when the filter advances, at which time it may go to 11.0 w.
2 Valve, 2-way, 2 position, continuous contact (21)	1	3 4	3 4	5	5	5		
3 Valves, 3-way, 2 position, momentary contact (5, 7, 10, 16, 17)	5	3 4	17 0	5	25	5		
4 Valve, 3-way, 3 position (9)	1	6 2	6 2	7	7	7		
5 Solenoid (enzyme)	1	2 0	2 0	2	2	5	5-7	
6 Reaction Unit-containing 3 reaction cylinders	1	11.0	11.0	10.5	10.5	0	Total All Valves	Built up of stainless steel and plastic
7 Tris Injection cylinders	1	2 0	2 0	3 0	3 0	0		
8 Enzyme storage unit	1	5 0	5 0	2 0	2 0	0		

Table No. 53 - Continued

COMPONENT	NO. UNITS	SIZE		WEIGHT		POWER		REMARKS
		PER UNIT in ³	TOTAL in ³	PER UNIT oz.	TOTAL oz.	PER UNIT w.	TOTAL w.	
9. Broth tank	2	4.0	8.0	3.0	6.0	0		
10. Culture chamber	2	4.0	8.0	2.0	4.0	0		
11. Coarse filter units	2	1.0	2.0	1.0	2.0	0		200 μ . or greater
12. Waste tank	1	2.0	2.0	1.0	1.0	0		
13. ATP reaction chamber	1	1.0	1.0	2.0	2.0	0		
14. Filter tape trans- port including motor, reels, tape, controls, etc.	1	80.0	80.0	48	48	10	10	size, weight, and power based on data for small air- borne tape recording units
15. TEA tanks	2	2.0	4.0	1.0	2.0	0		
16. Water tanks	1	12.0	12.0	6.0	6.0	0		
17. DMSO tank	1	2.0	2.0	1.0	1.0	0		
18. Tris tank	1	2.0	2.0	1.0	1.0	0		
19. Ammonium molybdate tank	1	2.0	2.0	1.0	1.0	0		
20. Oxygen tank	1	2.0	2.0	1.0	1.0	0		
21. Inert gas tank	1	16.0	16.0	16.0	16.0	0		
22. Check valves	16	0.5	8.0	0.5	8.0	0		
23. Tubing and fittings					10.0	0		

Table No. 53 - Continued

COMPONENT	UNITS	SIZE		WEIGHT		POWER		REMARKS
		PER UNIT in ³	TOTAL in ³	PER UNIT oz.	TOTAL oz.	PER UNIT w.	TOTAL w.	
24. Photomultiplier	1	4.0	4.0	10.0	10.0	0.5	0.5	operated at the same time, but not while filter tape is in motion
25. Signal processing electronics					1.0	1.0	1.0	
26. Control logic					1.0	1.0	1.0	solid state logic to cause sequential actuation of valves
27. Reagents					10.0			
28. Valve block to m- place items 1-4	1	7.0	7.0	18.0	18.0		7-10	by mounting all remotely actu- ated valves on one block and driving all with a single motor, gross valve weight can be cut greatly
TOTAL - max.		247.0		266.0			11.0	
min.		173.0		167.0			5.0	



The volume occupied by the system will be greater than the sum of the volumes of the components. However, even if the packing density were as poor as 50%, i.e., 50% of the total volume filled with hardware, it appears feasible to develop an AMML that occupies less than 346 cu. in. of space, weighs about 10 to 11 pounds and consumes 5 to 11 w. of electrical power 10 min. out of 30 at seven times separated by intervals of six or more hours, (i.e., less than 13 watt-hours of total energy).

VIII. CONCLUSIONS

A. Biochemistry

The biochemical phase of the present investigation has yielded very encouraging results, although in some areas, more work still has to be done to insure the confidence of the present life detection system. The following parameters for monitoring microbial metabolism through uptake of orthophosphate, sulfate and ATP production have been established.

1. A single medium, $\text{RM9-}^{35}\text{SO}_4$, can be used to detect phosphate uptake, sulfate uptake and ATP production by various microorganisms and soil cultures.
2. Through the inclusion of a poisoned control culture, along with the experimental culture, the presence of microorganisms can be detected by comparing the metabolic activities of the experimental and poisoned control cultures.
3. Direct inoculation of a prepared soil suspension as the microbial inoculum into the test medium is feasible.



4. Sulfate uptake is the most responsive detection system among three tested metabolic activities. In either pure or soil culture studies, sulfate uptake was generally recognized in a shorter period, while phosphate uptake and ATP production generally required a longer period of incubation to give pronounced results.

5. Static conditions and 26°C . of incubation were selected for growing the test organisms. The static condition provides the growth of both aerobic and anaerobic organisms, while the 26°C . was selected to simulate the highest likely diurnal temperature of the Martian atmosphere.

6. Areas to be improved include:

a. Enzyme storage problem

This problem is very important as far as ATP detection is concerned. A proper way to store the enzyme and to maintain maximum stability of the enzyme is needed.

b. Test of the present detection system on more microorganisms.

The Earth-born microorganisms comprise a great array of rather diversified organisms. Autotrophs, as well as anaerobic organisms and some organisms from extreme conditions, should be tested by the present system.

c. Test medium

A uniform test medium should be prepared and its stability during long storage periods should be tested.

d. Storage of reagents

Storage conditions, to avoid precipitation of reagents, or to avoid their interaction with the storing container should be studied.



e. Sterilization problem

The reagents, unless properly sterilized, cannot be used for the purpose of life detection. Methods for sterilization of the reagents, and especially of the enzyme, for flight mission require a special technique. More research is needed in this area.

B. Engineering

1. An automated microbial metabolism laboratory integrating the determination of phosphate uptake, sulfate uptake, adenosine triphosphate production, and carbon dioxide respiration is feasible.
2. Such an instrument, incorporating the phosphate and sulfate uptake, and ATP production and which would weigh not more than 5 kg., occupy not more than 6000 cm³ and require not more than 11 watts peak power, could be built within the current state of the art.
3. The following problem areas represent severe limitations and require further research and development:
 - a. The engineering aspects of pre-launch sterilization and cruise stability of the enzyme.
 - b. Miniaturization of reliable valving.
4. The following problem areas, while not representing severe limitations, require further study, preferably during the "breadboard" stage of developing the instrument:
 - a. Optimization of scintillation crystal and optical coupling for the photomultiplier.
 - b. Development of the filter tape and tape transport.



c. Choice of materials, especially for piping and chamber linings.

d. Development of the gas-driven syringes to meter and pump the fluid reactants.

e. Selection of a reliable flight-qualified photomultiplier tube.


IX. RECOMMENDATIONS

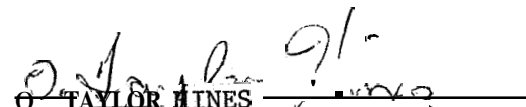
A. That additional research be conducted in the problem areas listed under Conclusions, (Section VIII. par. A6 and B3 above).

B. That this program be continued into the exploratory development phase, with the design and fabrication of an appropriate number of "breadboard" models, which would lead ultimately to the incorporation of these techniques into the Surface Laboratory of future planetary lander missions.

Submitted by


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REVISED SHEET

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APPENDIX I

THE HAZLETON INSTRUMENT

The laboratory apparatus referred to in the text as the "Hazleton Instrument" (to differentiate from other detectors reported in the literature as the "EMR Instrument," the "DuPont Instrument" and the "Wright-Patterson Instrument") is a light detector designed to be used with both bioluminescence and scintillation techniques. The detector consists of a photomultiplier tube (Fairchild-Dumont type **KM2485**) in a suitable housing to permit reading the light output of the ATP-luciferase-luciferin system and/or scintillations from various crystals excited by radioactive sources. Figure No. I-1 is a view of the instrument.

For sake of economy in both cost and overall size, and to match the size of the photocathode more nearly to the size of the ATP cell (about 1") and to the filter papers used for radioactive deposits (**13** and **25** mm.), it was decided to **use** a photomultiplier with a cathode of a nominal one-inch diameter. After a survey of commercially available tubes in this size, the RCA type 6199 and Dumont type **KM2485** were selected as the optimum choices for **S-11** and **5-20** responses respectively. The choice was based on a comparison of tube characteristics and costs. The characteristics of the tubes selected are listed in Table No. I-1 and more particularly, their responses are compared to firefly (ATP) and scintillator emissions in Figure No. **1-2**. Since the firefly peaks at a longer wavelength, the optimum match **was** the tube with the **S-20** response, i.e., **KM2485**.

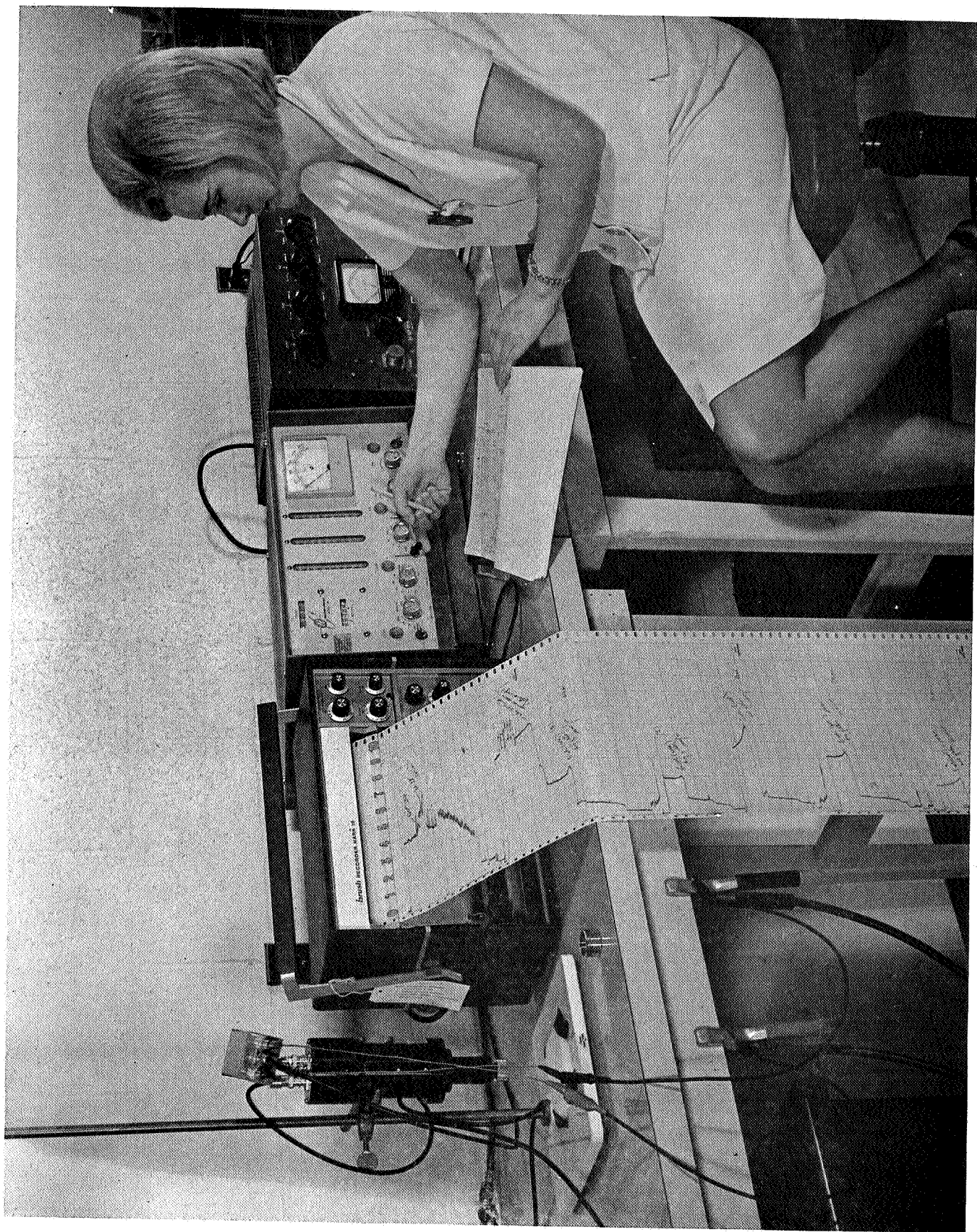


Figure 1-1 - Hazleton instrument for luminous detection

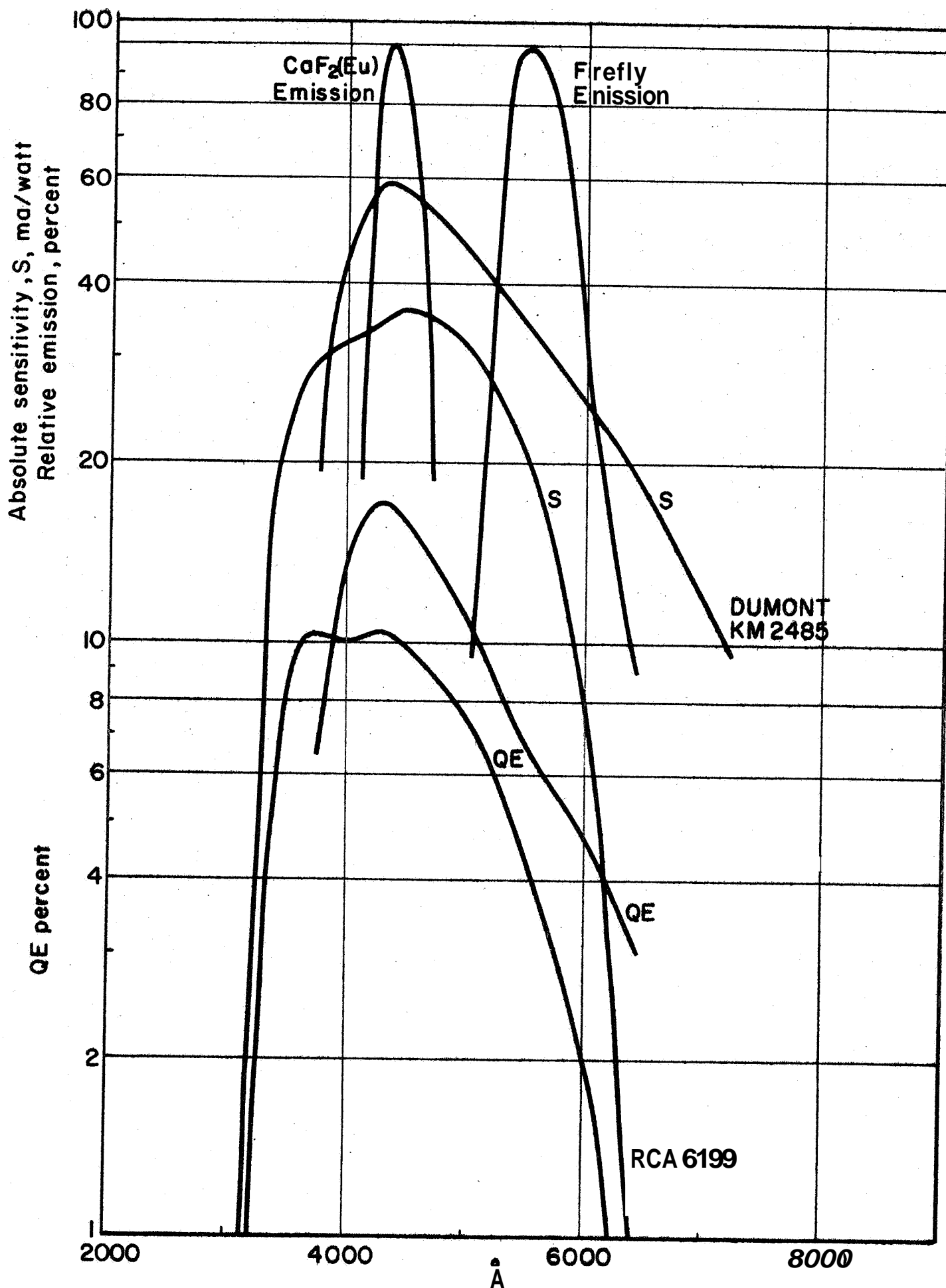


Figure No.1-2¹ - Characteristics of photomultiplier tubes

Table No. I-1 - Characteristics of
Photomultiplier tubes

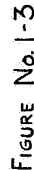
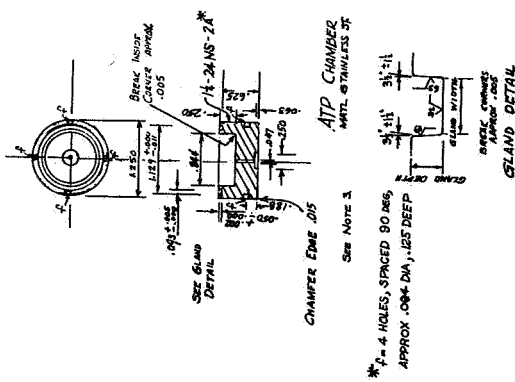
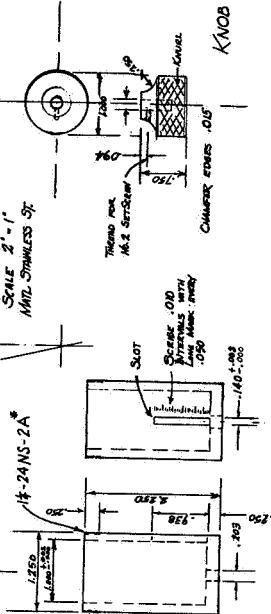
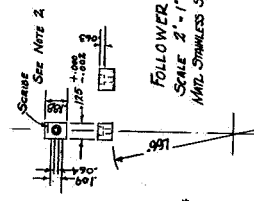
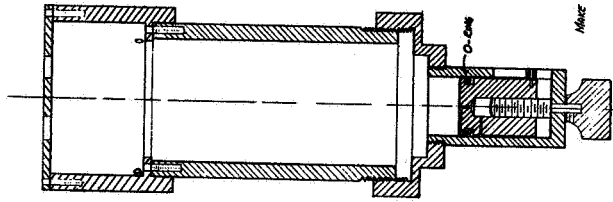
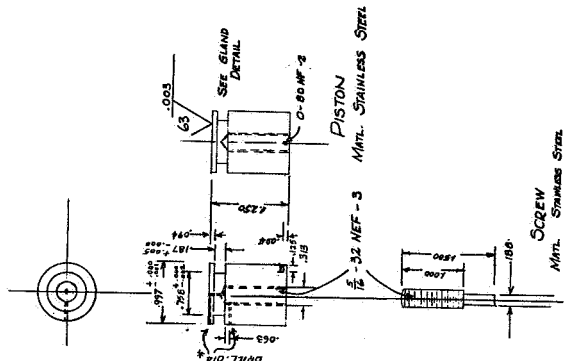
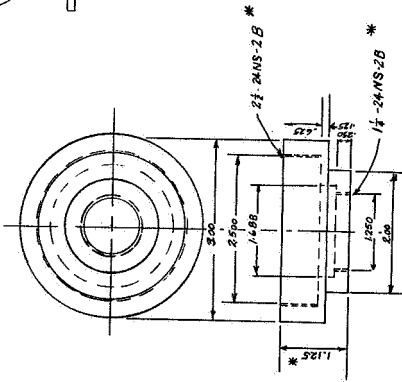
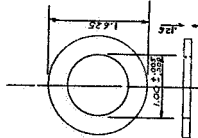
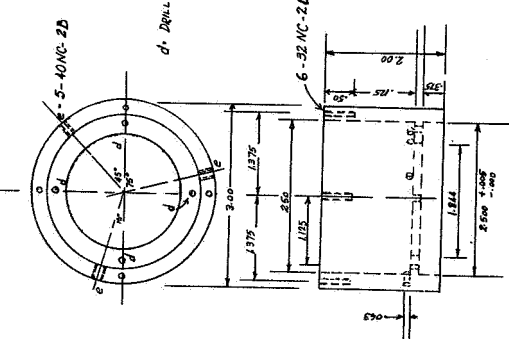
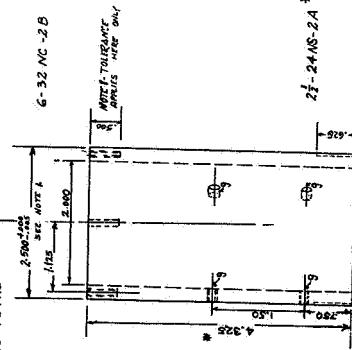
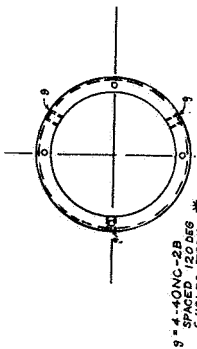
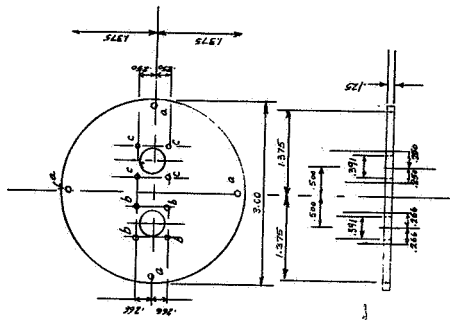
TYPE	UNIT	6199 (RCA)	KM2485 (DUMONT)
Response		S-11	5-20
Response @ 5600 Å	%	50	32
λ max. response	Å	4,400	4,200
DC supply voltage	v	1,000	1,800
Radiant sensitivity	a/w	21,600	23,200
Cathode sensitivity	a/w	.036	.058 min.
Current amplification		600,000	400,000
QE @ 5600 Å	%	4.3	7.8
Anode lum sensitivity	a/l	27	30
Cathode lum sensitivity	μa/l	45	130
Anode dark current (equiv.)	l.	2.5×10^9 max.	5×10^{-10}
Anode dark current (equiv.)	μA.	.005	.015 max.
Average anode current	ma.	0.75	1.0
Equiv. noise input	l.	4×10^{-12}	
Tube diameter	in.	$1\frac{1}{2}$	$1\frac{1}{4}$
Window diameter	in.	$1\frac{1}{4}$	1
Seated ht.	in.	3.88	4
Stages		10	10
Window index of refraction		1.51	
Cathode		Cs-Sb	multi-alkali
Ambient temp.	°C.	75	85

A housing was fabricated in accordance with drawings 604-112-1 and 604-112-2 (Figures No. 1-3 and No. 1-4). The tube socket and the associated voltage divider were mounted in the socket holder so that the tube, encased in its magnetic shield projected into the barrel with its face flush with the end of the barrel.

The assembly section shows the apparatus set up for scintillation tests. The assembly is supported vertically in a ring-stand, with the phototube face down. The desired scintillation crystal is placed in the crystal holder, which is then screwed onto the barrel. The filter paper carrying the radioactive precipitates is placed on top of the piston inside the sample cylinder, which is then screwed into the crystal holder. These operations must be performed in subdued light with the high voltage power OFF the tube. Otherwise irreparable damage may be done to the tube. Readings may then be made with the sample to crystal distance adjusted by the screw. The long thread on the crystal holder permits considerable variation in crystal thickness.

To make ATP tests, the crystal holder is removed and the chamber holder mounted in its place. A chamber is screwed into the chamber holder, using a round glass window (30 mm. dia. 2 mm. thick) with an O-ring on each side to provide a **seal**. The assembly is rotated to point the tube face-up for the test to provide better mixing. (It is returned to face-down when removing the chamber to avoid spilling the liquids on the tube). **The** reactants are injected into the chamber through the small hole, which has been plugged with a rubber compound ("Silastic"). This operation should be done in subdued light to minimize light leakage around the needle.

REV	DESCRIPTION	DATE	APPROVAL
1	CHANGES MARKED *	17 JUL 67	



NOTE 2: FOLLOWER FASTENED TO
POSTER WITH NO. 0-80 NF FLUJSTER
MACHINE SCREW. EXACT
LOCATION OF SCREW MARK
NOT IMPORTANT

NOTE 3: MARK 6; STAMP
IDENTIFYING NUMBERS (1,2,3,ETC) ON
FLAT FACE.

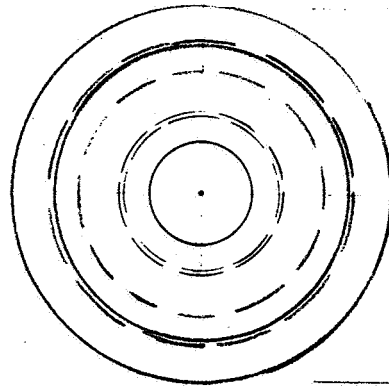
BLACK ANGOIZE AL PARTS

NOTE3: MAKE 6; STAMP
IDENTIFYING NUMBERS (1, 2, 3, ETC.) ON
FLAT FACE.

BLACK ANODIZE AL PARTS

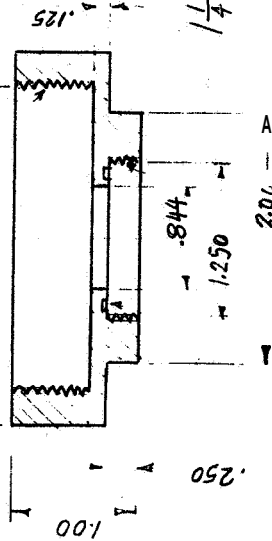
TYPE	COPY NO.	INDEX	DESCRIPTION	POTOMULTIPLIER	MOUNT	MATERIAL	AS ALLOY
							ELECT AS NOTED
HAZLETON LABORATORIES PALLS CHURCH, VIRGINIA							
						SHEET NO.	606-112-7
						DRAWING NO.	D
						SHEET 1 OF 1	REV. 1

Rev 1. Add Gland 26 JUL 67



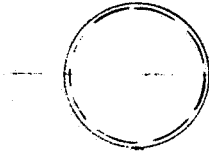
3.00
2.500

2 1/2 - 24 NS-2B



SEE GLAND DETAIL
DWG 604-112-1.

CHAMBER HOLDER



1.250
.250

1 1/4 - 24 NS-2A



KNURL

CHAMFER EDGE .015
PLUG

FIGURE No. 1-4

BLACK ANODIZE

B

ITEM	PART NO.	REQD.	DESCRIPTION	Photo Multiplier	Mounting	Comments	MATERIAL
LIST OF MATERIAL							
UNLESS OTHERWISE SPECIFIED DIMENSIONS ARE IN INCHES AND TOLERANCES ARE: .XX ± .03 .XXX ± .010 ANGLES : 2°							
NEXT ASSY	NAME	DATE					
DRAWN	PER	24 Jul 67					
CHECKED							
APPROVED							
APPROVED							
			MATL: AL. ALY.		SCALE: 1:1		
			DO NOT SCALE DRAWING				
			SHEET 1 OF 1 REV 1				

HAZLETON LABORATORIES	
FALLS CHURCH, VIRGINIA	
DWG NO.	604-112-2

The electronics are diagrammed in Figure No. 1-5 and listed in Table No. 1-2. For ATP determinations an oscilloscope (Techtronix 503) or a recorder (Brush 10) was connected across the load resistor (R12-R13). For beta counting, a scaler (Nuclear Chicago 186) was connected using the emitter-follower to drive the scaler.

Table No. 1-2 - Electronic parts list
for Hazleton Instrument

	-	1 meg., 1%, 1/4 w.
R2 through R11	-	500 K, 1%, 1/4 w.
R12 through R14	-	100 K, 5%, 1 w.
R15	-	10 K, 5%, 1/2 w.
C ₁	-	6 $\mu\mu\text{f.}$, 300 v.
C ₂	-	20 $\mu\mu\text{f.}$, 300 v.
C ₃	-	60 $\mu\mu\text{f.}$, 300 v.
C ₄	-	180 $\mu\mu\text{f.}$, 300 v.
PMT	-	Fairchild-Dumont Type KM2485